07-26-06 AF/ JFW

Docket No.: LATTA.002C4

July 25, 2006

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TRANSMITTAL LETTER
SUPPLEMENTAL APPEAL BRIEF

Applicant	:	Paul P. Latta
App. No	:	10/823,263
Filed	:	April 13, 2004
For	:	A METHOD OF TREATMENT OF DIABETES THROUGH INDUCTION OF IMMUNOLOGICAL TOLERANCE
Examiner	:	Belyavakyi, Michail
Art Unit	:	1644

CERTIFICATE OF EXPRESS MAILING

I hereby certify that this correspondence and all marked attachments are being deposited with the United States Postal Service as Express Mail No. **EV915029172US** in an envelope addressed to: Mai Stop Appeal Brief – Patents, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on

July 25, 2006
(Date)

Daniel E. Altman, Reg. No. 34,115

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Transmitted herewith for filing in the above-identified application are the following enclosures:

(X) Appeal Brief in 14 pages.

(X) 15 Appendices:

1. Specification as filed;
2. Office Action, mailed January 26, 2005;
3. USP 6,703,017, first cited by the Examiner in the Office Action, mailed January 26, 2005;
4. USP 5,425,764, first cited by the Examiner in the Office Action, mailed January 26, 2005;
5. USP 5,629,194, first cited by the Examiner in the Office Action, mailed January 26, 2005;

6. Posselt et al., "Promotion of pancreatic islet allograft survival by intrathymic transplantation of bone marrow" *Diabetes* 1992, **41**:771-775, cited by the Examiner in the Office Action, mailed January 26, 2005;

7. Summary of Interview conducted March 21, 2005;

8. Posselt et al. 1991 "Intrathymic islet transplantation in BB rats" *Ann. Surg.* **214**:363-373, provided by the Examiner at the Examiner's interview on March 21, 2005;

9. Response to Office Action of January 26, 2005, filed May 6, 2005 in 13 pages with Appendix I in 1 page and Appendix II, all made of record in the Office Action mailed June 16, 2005;

10. Appendix I - http://www.niaid.nih.gov/dait/NODmice.htm printout;

11. Appendix II - Hanninen et al. 2003 "Development of new strategies to prevent type I diabetes: the role of animal models" *Annals of Medicine* **35**:546-563;

12. Dr. D. Scharp Declaration under 37 CFR §1.132, in 9 pages with Exhibits 1-5 in 4 pages, Exhibit A in 32 pages, signed May 4, 2006, filed May 6, 2005 and acknowledged by the Examiner in the Office Action mailed June 16, 2005;

13. Office Action, mailed June 16, 2005;

14. Response to Office Action of June 16, 2006, filed August 17, 2005 and made of record in the Final Office Action mailed November 3, 2005;

15. Final Office Action, mailed November 3, 2005.

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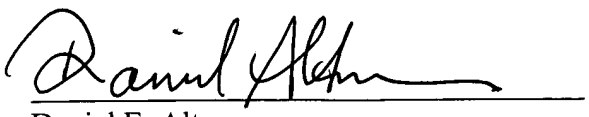
Dated: July 25, 2006



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Please Direct All Correspondence to Customer Number **20995**

SUPPLEMENTAL APPEAL BRIEF

Applicant	:	Paul P. Latta
App. No	:	10/823,263
Filed	:	April 13, 2004
For	:	A METHOD OF TREATMENT OF DIABETES THROUGH INDUCTION OF IMMUNOLOGICAL TOLERANCE
Examiner	:	Belyavskyi, Michail
Art Unit	:	1644

Mail Stop Appeal Brief-Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In accordance with the Notice of Appeal filed February 3, 2006, and in response to the Notification of Non-Compliant Appeal Brief, mailed June 26, 2006, Applicant submits this Supplemental Appeal Brief.

TABLE OF CONTENTS

I. REAL PARTY IN INTEREST

Pursuant to 37 C.F.R. §1.192, Appellants hereby notify the Board of Patent Appeals and Interferences that the real party in interest is the inventor for this application, Paul P. Latta, 33 Santa Cruz Aisle, Irvine, CA 92606.

II. RELATED APPEALS AND INTERFERENCES

Appellants are currently appealing to the US Board of Patent Appeals and Interferences in a related case, No.: 10/660,924.

III. STATUS OF CLAIMS

The above-identified application was filed with 14 claims. In response to the Office Action mailed June 16, 2005, claim 12 was cancelled and Claims 1, 6, and 11 were amended. Claims 1-11, 13 and 14 were finally rejected by the Examiner in the Final Office Action mailed November 3, 2005. Accordingly, Claims 1-11, 13 and 14 are the subject of this appeal. The Claims are attached hereto as Appendix VIII.

IV. STATUS OF AMENDMENTS

No amendments have been filed subsequent to the final rejection.

V. CONCISE EXPLANATION OF SUBJECT MATTER OF INDEPENDENT CLAIM

Claim 1 is the only independent claim on this appeal. The subject matter of this claim relates to Appellant's discovery of a method of treating of Type I diabetes in a mammal. See Applicant's specification at page 8, line 2 through page 9, line 11. The method includes as a first step implanting a dose of insulin-producing cells into an implantation site in said mammal. See page 10, lines 22-24. The cells that are implanted are encapsulated in a biologically-compatible membrane. See page 3, lines 30-31 and page 8, lines 3-29. The implantation site is subcapsular, subcutaneous, intraperitoneal or intraportal. See page 4, lines 16-17. In the second step of the claimed method a fully therapeutic dose of un-encapsulated insulin-producing cells is implanted. See page 8, line 30 through page 9, line 1, and page 17, lines 12-15.

When using the method of this claim, the encapsulated cells shed antigens through the capsule membrane into the blood stream of a host, while being protected from the attack by the host immune system. Over time, the host immune system gets tolerized to the implanted cells and when the fully therapeutic dose of the same cells is implanted, it is recognized by the immune system as "self" and a rejection response is not elicited. See page 9, lines 1-4 and page 17, lines 1-11.

Each limitation of Claim 1, the only independent claim, is supported by the Specification as filed as follows:

LIMITATION	PAGE:LINES
A method of treating diabetes in a mammal in need thereof, comprising the steps of:	8:2 through 9:11
implanting in said mammal a tolerizing dose of insulin-secreting cells	10: 22-24
encapsulated in a biologically compatible permselective membrane,	3:30-31; 8: 3-29
wherein said implanting step is subcapsular, subcutaneous, intraperitoneal or intraportal; then	4:16-17
administering to said mammal a therapeutic dose of corresponding unencapsulated insulin-secreting cells.	8:30 through 9:1; 17:12-15

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

1. The Examiner has rejected Claims 1-4, 6-11, 13 and 14 under 35 U.S.C. §103(a), as being obvious over USP 6,703,017, or by USP 5,425,764 or USP 5,629,194 each in view of Posselt et al. (*Diabetes*, 1992, **41**:771-775)[1].

2. The Examiner has also rejected Claim 5 under 35 U.S.C. §103(a), as being obvious over USP 6,703,017, or by USP 5,425,764 or USP 5,629,194 each in view of Posselt et al. (*Diabetes*, 1992, **41**:771-775) as applied to claims 1-4 and 6-14, and further in view of USP 5,529,914[2].

[1] Final OA, mailed 11/03/05, page 2, paragraph 3.
[2] *Id.*, at page 5, paragraph 4.

VII. ARGUMENT

1. The Examiner has improperly rejected Claims 1-4, 6-11, 13 and 14 as obvious.

> Pursuant to MPEP 2143, in order to establish a *prima facie* case of obviousness three requirements must be met: First there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations.

None of the three primary references cited by the Examiner provide any teaching of a tolerizing dose followed by a therapeutic dose. All three of these patents teach only the administration of a single fully-therapeutic dose. For example, U.S. Patent 6,703,017 describes treating diabetes in a mammal by creating a pancreas-like structure in a human patient. The patent indicates that "from data relating to transplantation of ex vivo islets in humans, it is expected that about 8,000-12,000 IdIs per patient kg may be required" to achieve the desired therapeutic effect.[3] The implant in USP '017 is designed to treat diabetes by creating a live "insulin pump" in the body. Example 12 of USP '017 describes implanting 5,000 islets per NOD mouse, which is equivalent to 200,000 islet/kg of body weight. This treatment resulted in normoglycemia in these animals[4]. Thus, the '017 patent teaches the administration of only one fully-therapeutic dose of insulin-producing cells. It does not teach or suggest first implanting a tolerizing dose of insulin-producing cells prior to implanting the fully-therapeutic dose, wherein the tolerizing dose is at least one order of magnitude less than the therapeutic dose.

US Patent 5,425,764 describes a method of using an implantable bioartificial pancreas device containing insulin-secreting islets, to supply an exogenous source of insulin to treat the symptoms of diabetes[5]. Accordingly, the '764 patent also requires implantation of a fully therapeutic dose of insulin-secreting cells, i.e. the dose necessary to achieve normoglycemia. As such, the '764 Patent does not describe or suggest implanting a tolerizing dose of insulin-

[3] USP 6,703,017, col. 14, lines 7-9.

[4] *Id.* col. 23, line 48 through col. 24, line 5.

producing cells prior to implanting of a fully therapeutic non-encapsulated dose, where the tolerizing dose is at least one order of magnitude less than the therapeutic dose.

Similarly, US Patent 5,629,194 describes a method of implanting embryonic porcine pancreatic non-insulin-secreting cells capable of proliferating *in vivo* and then secreting insulin after transplantation. The dose sufficient for the treatment of insufficient insulin activity is 100,000-500,000 aggregates, each containing 300-500 cells per human patient[6]. This is a fully therapeutic dose. Thus, like the other two primary references, the '194 patent does not describe or suggest implanting a tolerizing dose of insulin-producing cells prior to implanting of fully therapeutic non-encapsulated dose, where the tolerizing dose is at least one order of magnitude less than the therapeutic dose.

The Examiner relied on a reference of Posselt et al. to show the administration of a tolerizing dose. In the Office Action mailed January 26, 2005, the Examiner cited Posselt et al., *Diabetes* 1992, **41**:771-775[7] (Posselt 1992). However, during the Examiner's interview conducted March 21, 2005[8], it was agreed that the correct reference should have been Posselt et al., *Ann. Surg.* 1991 **214**:363-373[9] (Posselt 1991). Nevertheless, in the Office action mailed June 16, 2005[10], and in the Final Office Action, mailed November 3, 2005, the Examiner again cited the Posselt 1992, reference[11]. It is clear that this is the wrong reference because in setting forth the bases for the rejection, the Examiner referred to the page numbers from the Posselt 1991 reference[12]. Therefore, Appellant herein discusses the correct reference of Posselt 1991.

In the Final Office Action, the Examiner stated that "Posselet (sic) et al., teach two step strategy: first administering a small dose of cell that induces an unresponsive state, i.e. tolerizing dose and then administering fully therapeutic dose, at another site." The Examiner further states

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[5] USP 5,425,764 col. 2, lines 53-55; col. 6, lines 8-14; and claims 1-31.
[6] USP 5,629,194, col. 13, lines 12-28.
[7] Office Action, mailed 01/26/2005, page 5, paragraph 10.
[8] Summary of Interview conducted March 21, 2005.
[9] Amendment filed May 6, 2005 (Summary of Interview).
[10] Office Action, mailed 06/16/2005, page 2, paragraph 3.
[11] Final Office Action, mailed 11/03/05, page 2, paragraph 3.
[12] *Id.*, page 3, line 3.

that "[t]here is no indication or suggestion in Posselt et al. that only intrathymic transplantation should be performed." And further: "it is noted that the instant claims does (sic) not recited (sic) any specific place where a first tolerizing dose of insulin-secreting cells should be implanting (sic)."[13] All of these statements are clearly incorrect.

First, although Posselt 1991 does disclose a two-step process of administering insulin-producing cells, the initial tolerizing dose is not "one to two orders of magnitude less than [a] therapeutic dose" as recited in pending Claim 3. The only doses of insulin-producing cells described in Posselt 1991 are described on page 364 of the reference under the heading "*Islet Isolation and Transplantation.*" 1000 to 1500 islets were transplanted into the liver and renal subcapsule, and 600 to 800 islets were transplanted into each lobe of the thymus, for a total of 1200 to 1600 islets. In the rats that did not destroy these islets, "serum glucose levels in these animals returned to normal within 48 to 72 hours after islet transplantation."[14] Thus, the dose that was applied was a fully therapeutic dose, rather than the tolerizing dose one to two orders of magnitude less than a therapeutic dose recited in Claim 3.

Moreover, the Examiner disagreed with Appellant's characterization of the Posselt 1991 reference as teaching away from delivery of the initial dose of insulin-producing cells to any site other than the Thymus. However, as explained below, one of ordinary skill in the art reviewing the Posselt 1991 reference would clearly conclude that the initial dose must be given to the thymus.

Posselt 1991 describes implanting unencapsulated islets into various areas of the body, including liver, kidney, and thymus, of spontaneously diabetic BB rats[15]. The only implantation site that showed survival of the implanted cells was the thymus[16]. The islets injected into the liver were rejected almost immediately, while islets injected into the kidney capsule had variable

[13] *Id.*, page 2, last paragraph through page 3, first paragraph.
[14] Posselt et al.1991 "Intrathymic islet transplantation in BB rats" *Ann. Surg.* **214**:363-373 at page 365 left column.
[15] *Id.* at page 363, left column.
[16] *Id.* page 364, right column through page 365, right column.

survival, with only two surviving as long as 120 days.[17] In contrast, the intrathymic islet recipients were observed for a period close to the life span of the rat, without any recurrent diabetes. There is no indication of any kind in the Posselt 1991 reference that any site other than thymus can be used to induce immunological tolerance. Indeed, the authors state several times in this article that thymus is considered to be an immunologically privileged site.[18]

In experiments conducted on intrathymically injected animals, approximately 100 days after the initial intrathymus transplantation, the transplanted rats were challenged with extrathymic allogeneic islets, which remained intact even after removal of the thymus bearing the islet allografts[19]. However, in animals which were able to maintain functional subcapsular islets for more than 120 days, the vigor of the immune response to subsequent allografts was not diminished[20]. As the authors stated several times in this article, thymus is considered to be an immunologically privileged site and is subject to the usual biologic characteristics of such sites, in that prior sensitization of the host with skin allografts precludes prolonged survival of intrathymic islets[21]. The experiments, performed by Posselt et al. show just that, i.e. when allogeneic islets were transplanted into the thymus of recipients that had previously rejected donor strain skin grafts, the islets were destroyed in an accelerated manner, demonstrating that the intrathymic site is readily accessible to activated T cells[22], and that no tolerance can be achieved using this protocol. Furthermore, Posselt et al. goes on stating that the achieved tolerization to intrathymic allografts is due to their direct influence on maturing thymocytes, which are more susceptible to tolerance-inducing signals, and that such "inappropriate" presentation of antigen by nonlymphoid cells induce a state of anergy in T cells[23].

Therefore, Posselt et al. clearly do teach away from using the initial dose of insulin-producing cells anywhere but thymus, and it only shows success in the absence of prior

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[17] *Id* page 365, left column.
[18] *Id.* page 372, right column.
[19] *Id.* page 365, right column through page 366, left column.
[20] *Id.*, page 367, left column.
[21] *Id.* page 372, right column.
[22] Id. page 367 left column, and page 368, right column.
[23] Id. page 373, both columns.

sensitization to the implant. If a skilled artisan was still looking for a way to solve the problem of creating tolerance to the implant other than described in the USP'017, USP'764, and USP'194, the publication of Posselt 1991 would point the artisan only in one direction: intrathymic implantation of a tolerizing dose of insulin-producing cells, because this reference convincingly teaches away from using tolerizing dose of insulin-producing cells anywhere but thymus, and it does not teach encapsulating these cells. Therefore, contrary to the Examiner's assertion, one skilled in the art would have no reasonable expectation of success in using the invention of the presently recited claims involving subcapsular, subcutaneous, intraperitoneal or intraportal implantation, and would have no motivation to do so after reading Posselt 1991.

Furthermore, the Examiner's statement that "the instant claims does (sic) not recited (sic) any specific place where a first tolerizing dose of insulin-secreting cells should be implanting (sic)" is simply inaccurate. All of the claims recite that the "implanting step is subcapsular, subcutaneous, intraperitoneal or intraportal." Thus, these claims clearly exclude implantation into the thymus.

Furthermore, as Dr. Scharp states in his Declaration submitted on May 6, 2005, the BB rat used as a model in Posselt 1991 has multitude of immunologic disorders that makes it more of a model for immune deficiency than a model for diabetes. Therefore, the BB rat is no longer considered an acceptable model for studying human autoimmune diabetes[24]. This is also stated by Posselt 1991: "BB rats are known to be significantly immunodeficient"[25].

The Applicant was the first one to teach that implantation of insulin-producing cells in sites other than thymus produces tolerance to the implanted cells. The Declaration of Dr. Scharp, submitted on May 6, 2005, reiterates that point.[26] Contrary to the Examiner's statement, the instant method as claimed in the present Claims 1-14 specifies the non-thymus implantation sites for the tolerizing dose of encapsulated cells. This limitation is not suggested in any of the cited references. Therefore, the cited references fail to suggest the claimed method. Accordingly,

[24] Declaration of Dr. Scharp, page 3, paragraph 8.
[25] Posselt et al. 1991 "Intrathymic islet transplantation in BB rats" Ann. Surg. **214**:363-373 page 365, left column.
[26] Declaration of Dr. Scharp, page 3, paragraph 10 through page 4, paragraph 16.

even when combined, these references do not teach all the limitations of the claimed invention. As such, the cited references fail to support a *prima facie* case of obviousness.

Additionally, with regard to Claim 3, none of the references including Posselt 1991 disclose or suggest an initial administration of a dose of cells less than a fully therapeutic dose. Accordingly, Claim 3 is nonobvious for this additional reason.

Finally, the claims recite that the insulin-producing cells are "encapsulated in a biologically compatible permselective membrane." One of ordinary skill in the art would not expect to inject such encapsulated cells into the thymus as suggested by Posselt 1991 because of the small size of the thymus.[27] Thus, one of ordinary skill in the art would not expect to be able combine the recited encapsulated cells with the methods of the three primary references and Posselt 1991. Thus, because the necessary motivation, teaching or suggestion to combine the references is absent, there is no *prima facie* case of obviousness for this additional reason as well.

Therefore, Claims 1-4 and 6-14 are in compliance with 35 U.S.C. 103(a), and the rejection of Claims 1-4, 6-11, 13 and 14 as obvious is clearly improper.

2. The Examiner has improperly rejected Claim 5 as being obvious. Non-obviousness of the independent Claim 1 in view of US Patent 6,703,017 or by US Patent 5,425,764, or US Patent 5,629,194 each in view of Posselt 1991 is asserted above. US Patent 5,529,914 discloses a method of encapsulating cells, but it fails to cure the deficiencies of US Patent 6,703,017, US Patent 5,425,764, US Patent 5,629,194, and Posselt et al. Therefore, Claim 5 is in compliance with 35 USC §103(a), and the rejection of Claim 5 as obvious is clearly improper.

[27] A typical adult human thymus has a volume of 12 ml.

Conclusion

In view of the arguments presented above, Appellants submit that the Specification as filed enables a person with an ordinary skill in the art on how to make and use the invention. Appellants further submit that Claims 2-9 are fully supported by the Specification as filed and do not constitute New Matter.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP.

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071306

VIII. CLAIMS APPENDIX

1. **(Previously presented)** A method of treating diabetes in a mammal in need thereof, comprising the steps of:

implanting in said mammal a tolerizing dose of insulin-secreting cells encapsulated in a biologically compatible permselective membrane, wherein said implanting step is subcapsular, subcutaneous, intraperitoneal or intraportal; then

administering to said mammal a therapeutic dose of corresponding unencapsulated insulin-secreting cells.

2. **(Original)** The method of claim 1, wherein said mammal is a human, canine or feline.

3. **(Previously presented)** The method of claim 1, wherein said tolerizing dose is one to two orders of magnitude less than said therapeutic dose.

4. **(Original)** The method of claim 1, wherein said insulin-secreting cells are pancreatic islet cells.

5. **(Original)** The method of claim 1, wherein said membrane comprises polyethylene glycol.

6. **(Previously presented)** The method of claim 1, wherein said tolerizing and therapeutic doses comprise porcine cells.

7. **(Previously presented)** The method of claim 1, further comprising the step of administering one or more anti-inflammatory agents to said mammal prior to, at the same time as, or subsequent to administration of said therapeutic dose.

8. **(Original)** The method of claim 1, wherein said membrane has a molecular weight cutoff of about 150 kDa or less.

9. **(Original)** The method of claim 1, wherein said membrane has a pore size of less than about 0.4 μm.

10. **(Original)** The method of Claim 9, wherein said membrane has a pore size of less than about 0.2 μm.

11. **(Previously presented)** The method of Claim 1, wherein said therapeutic dose is between one and two orders of magnitude higher than said tolerizing dose.

12. **(Cancelled)**

13. **(Original)** The method of Claim 1, wherein said administering step is intraperitoneal, intraportal or subcutaneous.

14. **(Original)** The method of Claim 1, wherein said tolerizing dose is administered incrementally.

IX. EVIDENCE APPENDIX

1. Specification as filed;
2. Office Action, mailed January 26, 2005;
3. USP 6,703,017, first cited by the Examiner in the Office Action, mailed January 26, 2005;
4. USP 5,425,764, first cited by the Examiner in the Office Action, mailed January 26, 2005;
5. USP 5,629,194, first cited by the Examiner in the Office Action, mailed January 26, 2005;
6. Posselt et al., "Promotion of pancreatic islet allograft survival by intrathymic transplantation of bone marrow" *Diabetes* 1992, **41**:771-775, cited by the Examiner in the Office Action, mailed January 26, 2005;
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12. Dr. D. Scharp Declaration under 37 CFR §1.132, in 9 pages with Exhibits 1-5 in 4 pages, Exhibit A in 32 pages, signed May 4, 2006, filed May 6, 2005 and acknowledged by the Examiner in the Office Action mailed June 16, 2005;
13. Office Action, mailed June 16, 2005;
14. Response to Office Action of June 16, 2006, filed August 17, 2005 and made of record in the Final Office Action mailed November 3, 2005;
15. Final Office Action, mailed November 3, 2005.

X. RELATED PROCEEDINGS APPENDIX

There are no decisions rendered by a court or the Board in any related proceedings identified above.

LATTA.002C4

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Latta, Paul P.
Appl. No.	:	10/823,263
Filed	:	April 13, 2004
For	:	A METHOD OF TREATMENT OF DIABETES THROUGH INDUCTION OF IMMUNOLOGICAL TOLERANCE (as amended)
Examiner	:	Belyavski, Michail A.
Group Art Unit	:	1644

CERTIFICATE OF MAILING

I hereby certify that this correspondence and all marked attachments are being deposited with the United States Postal Service as first-class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on **May 6, 2005**.

Daniel Altman

Daniel Altman, Reg. No. 34,115

AMENDMENT

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In response to Office Action mailed January 26, 2005, and interview conducted March 21, 2005 please amend the above-identified application as follows:

Amendments to the Specification begin on page 2 of this paper.

Amendments to the Claims are reflected in the listing of claims which begins on page 3 of this paper.

Summary of Interview begins on page 5 of this paper.

Remarks/Arguments begin on page 6 of this paper.

AMENDMENTS TO THE SPECIFICATION

Please replace the Title of the Invention with the following title:

A METHOD OF TREATMENT OF DIABETES THROUGH INDUCTION OF IMMUNOLOGICAL TOLERANCE

AMENDMENTS TO THE CLAIMS

1. **(Currently amended)** A method of treating diabetes in a mammal in need thereof, comprising the steps of:

implanting in said mammal a tolerizing dose of insulin-secreting cells ~~from the same species as said mammal~~ encapsulated in a biologically compatible permselective membrane; then

administering to said mammal a ~~curative~~ therapeutic dose of corresponding unencapsulated insulin-secreting cells.

2. **(Original)** The method of claim 1, wherein said mammal is a human, canine or feline.

3. **(Currently amended)** The method of claim 1, wherein said tolerizing dose is one to two orders of magnitude less than said ~~curative~~ therapeutic dose.

4. **(Original)** The method of claim 1, wherein said insulin-secreting cells are pancreatic islet cells.

5. **(Original)** The method of claim 1, wherein said membrane comprises polyethylene glycol.

6. **(Currently amended)** The method of claim 1, wherein said tolerizing and ~~curative~~ therapeutic doses ~~are~~ comprise porcine cells.

7. **(Currently amended)** The method of claim 1, further comprising the step of administering one or more anti-inflammatory agents to said mammal prior to, at the same time as, or subsequent to administration of said ~~curative~~ therapeutic dose.

8. **(Original)** The method of claim 1, wherein said membrane has a molecular weight cutoff of about 150 kDa or less.

9. **(Original)** The method of claim 1, wherein said membrane has a pore size of less than about 0.4 μm.

10. **(Original)** The method of Claim 9, wherein said membrane has a pore size of less than about 0.2 μm.

11. **(Currently amended)** The method of Claim 1, wherein said ~~curative~~ therapeutic dose is between one and two orders of ~~magniture~~ magnitude higher than said tolerizing dose.

12. **(Original)** The method of Claim 1, wherein said implanting step is subcapsular, subcutaneous, intraperitoneal or intraportal.

13. **(Original)** The method of Claim 1, wherein said administering step is intraperitoneal, intraportal or subcutaneous.

14. **(Original)** The method of Claim 1, wherein said tolerizing dose is administered incrementally.

SUMMARY OF INTERVIEW

Exhibits and/or Demonstrations

Experimental data showing that implanting a tolerizing (sub-therapeutic) dose of encapsulated insulin-producing cells into NOD mice prior to the animals developing diabetes protected these animals from diabetes for the rest of their natural lives as shown by their normoglycemia and lack of insulitis.

Identification of Claims Discussed

1-14

Identification of Prior Art Discussed

USP 6,703,017; 6,425,764; Posselt et al. Diabetes 1992 41:771-775.

Proposed Amendments

None

Principal Arguments and Other Matters

The Applicant argued that claims 1-14 are non-obvious over USP 6,703,017 and 6,425,764 in view of Posselt et al. *Diabetes* 1992 **41**:771-775.

Results of Interview

Applicant will provide Declaration showing the histology of the mice that were prevented from developing Type I diabetes. Applicant agreed to consider amending claims. The Examiner provided the correct copy of the Posselt et al. reference (i.e. 1991 *An. Surg.* **214**:363-373).

REMARKS

Claims 1-14 are pending. Claims 1, 3, 6, 7, and 11 have been amended. Support for the amendments can be found in the Specification as filed, for example, 8:30-31 and 9:26-27. The following addresses the substance of the Office Action and the Examiner Interview.

1. Title of the invention is not descriptive

The Examiner has requested amending the Title of the Invention to clearly indicate the invention to which the claims are directed. Applicant has now amended the Title accordingly.

2. References in IDS not found in File

Applicant has resubmitted the references previously submitted in the parent application in Applicant's co-pending Application No. 10/660,924 of which the present application is a continuation. Accordingly, pursuant to 37 C.F.R. 1.98(d), additional copies of the references are not submitted in the present application.

3. Compliance with 35 USC §112, second paragraph

The Examiner has rejected Claims 1-14 under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. More specifically, the examiner has stated that there is insufficient antecedent basis for the limitation "curative" in the claim. During the interview on March 21, 2005, the Examiner indicated that the use of the term "therapeutic" would be acceptable. Applicant has now amended claims 1, 3, 6, 7, and 11 to recite the term "therapeutic" instead of "curative". Support for this amendment can be found on page 8, line 30-31 of the Specification as filed. Claim 6 was additionally rejected as being indefinite and ambiguous in the recitation of "wherein said tolerizing and curative doses are porcine", because "doses" can not be porcine. Applicant has amended claim 6 to read "wherein said tolerizing and therapeutic doses comprise porcine cells." Support for this amendment can be found in the Specification as filed, page 9, lines 26-27. Therefore, Claims 1-14 are now in compliance with 35 USC §112, second paragraph.

4. Compliance with 35 USC §112, first paragraph

The Examiner has rejected Claims 1-14 under 35 U.S.C. 112, first paragraph, as being not in compliance with the enablement requirement. The Examiner stated that the specification does not reasonably provide enablement for a method of treating Type I diabetes in a mammal

comprising implanting a tolerizing dose of insulin-producing cells encapsulated in a biologically compatible membrane and then administering a curative dose of corresponding unencapsulated insulin-secreting cells. According to MPEP 2164:

> "The test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation." A patent need not teach, and preferably omits, what is well known in the art."(2164.01)
>
> There are many factors to be considered when determining whether there is sufficient evidence to support a determination that a disclosure does not satisfy the enablement requirement and whether any necessary experimentation is "undue." These factors include, but are not limited to: (A) The breadth of the claims; (B) The nature of the invention; (C) The state of the prior art; (D) The level of one of ordinary skill; (E) The level of predictability in the art; (F) The amount of direction provided by the inventor; (G) The existence of working examples; and (H) The quantity of experimentation needed to make or use the invention based on the content of the disclosure. "not everything necessary to practice the invention need be disclosed. In fact, what is well-known is best omitted. All that is necessary is that one skilled in the art be able to practice the claimed invention, given the level of knowledge and skill in the art."

Here, the scope of the claims is a method of inducing tolerance to insulin-producing cells implanted in an animal, including human, for the therapeutic result of treating diabetes. Every element of claim 1 is well within the level of knowledge of a skilled artisan, e.g., implanting foreign cells is common practice by medical doctors specializing in transplants; encapsulating methods are also well-known to a person skilled in the art, and Patent 5,529,914 which has been incorporated by reference into the Specification as filed teaches methods of encapsulating cells for implantation; calculating tolerizing dose and therapeutic dose for a specific mammal is disclosed in Examples 1 to 7 as "one or two orders of magnitude less than a curative dose". These examples give the tolerizing dose and therapeutic dose for several important medical conditions as follows:

Disease	Cells	Tolerizing Dose	Curative (Therapeutic) Dose	Support in the Specification
Diabetes	Islets/insulin producing cells	100-2,000 islets/kg of body weight 1,500 islets/kg of body weight	10,000-20,000 islets/kg of body weight 15,000 islets/kg of body weight	12:26-30 25:13-16
Parkinson's	Adrenal Chromaffin cells	1,000 cells/kg of body weight	10,000 cells/kg of body weight	25:21-26
Hemophilia	Liver cells	2,500 cells/kg of body weight	5,000 cells/kg of body weight	25:29-26:5

Disease	Cells	Tolerizing Dose	Curative (Therapeutic) Dose	Support in the Specification
Liver Transplant	Liver cells	1,000 cells/kg of body weight	Whole liver	26:7-11
Myasthenia gravis	Neural cells expressing acetylcholine receptor	2,500 cells/kg of body weight		26:13-19
General		Between about 100 cells/kg body weight and about 5,000 cells/kg body weight	Between about one and two orders of magnitude higher than tolerizing dose	13:1-4

Based on the guidance provided, a person skilled in the art would immediately know the dose required for their patient depending on the condition to be treated. Medical doctors routinely calculate dosages for patients by considering such factors, including but not limited to, weight, age, sex, degree of disease, etc. A curative (therapeutic) dose of implanted islets is well known in the field and a person skilled in the arts could readily calculate a one to two magnitude decrease in this dose to obtain the numbers for the tolerizing dose.

The specification also provides experimental proof of principle, i.e. experimental data showing that an encapsulated insulin-producing cells given as a small mass insufficient by itself to induce normoglycemia permits a second, unencapsulated, implant of insulin-producing cells in a therapeutic dose to survive as shown by normalized blood glucose levels in the treated mice in which diabetes had been induced by intravenous injection of streptozotocin. See Example 1.

Nevertheless, the Examiner is questioning the value of such evidence based on alleged lack of predictability of the treatment of diabetes in human from *in vivo* data obtained in murine models of diabetes. However, the Examiner is setting forth a much stricter standard than required by law. MPEP 2107.03 establishes the following:

> Evidence does not have to be in the form of data from an art-recognized animal model for the particular disease or disease condition to which the asserted utility relates. Data from any test that the applicant reasonably correlates to the asserted utility should be evaluated substantively. Thus, an applicant may provide data generated using a particular animal model with an appropriate explanation as to why that data supports the asserted utility. The absence of a certification that the test in question is an industry-accepted model is not dispositive of whether data from an animal model is in fact relevant to the asserted utility. Thus, if one skilled in the art would accept the animal tests as being reasonably predictive of utility in humans, evidence from those tests should be considered sufficient to

support the credibility of the asserted utility."

In the Declaration by David Scharp, M.D. submitted under 37 CFR §1,132, additional data is presented which shows that the claimed method works in two murine models of diabetes: the streptozotocin-induced diabetic mice and in non-obese diabetic (NOD) mice. These are the two well-known and widely accepted murine models of diabetes. The multiple low-dose streptozotocin (MLDS) model of diabetes is characterized by progressive hyperglycemia and insulitis similar to that observed in recent onset type I diabetics (Like and Rossini, 1976 *Science* **193**:415-417). The NOD mouse model also shares clinical serological and histo-immunological features with human type I diabetes (Bach, 1994 *Endocrine Rev.* **15**:516-542). As in humans, the disease is characterized by infiltration of the pancreatic islets by immune cells, insulitis followed by destruction of the β-cells. Both models have been used extensively to study new therapies for diabetes. In fact, the NIH recognize the NOD mouse as THE model animal for diabetes and maintains a research colony and data base on these animals for researchers. The NIH state "The NOD mouse, which spontaneously develops type 1 diabetes, is a valuable animal model that is used extensively in research exploring the etiology, prevention, and treatment of this disease. It is a vital research tool for testing promising prevention and treatment strategies at the preclinical level." (http://www.niaid.nih.gov/dait/NODmice.htm, copy attached herein).

The evidence in the Declaration reiterates the results provided in the specification showing that the claimed method is effective to permit survival of a therapeutic dose of insulin-producing cells and thereby effectively treating diabetes. *See* Declaration ¶ 6. Moreover, the Declaration also establishes that implantation of a sub-therapeutic, tolerizing dose of insulin-secreting cells is effective to create immunological tolerance to insulin-secreting cells, namely the host's own islet cells. Together these results establish that the claimed method of treating diabetes by tolerizing the host immune system prior to implanting the fully therapeutic dose of the insulin-producing cells works to permit the host to receive the fully therapeutic dose without rejection. *See* Declaration ¶¶ 7-16.

Therefore, using the proper standard set forth in the MPEP, the evidence provided by Applicant in the Specification and in the Declaration submitted herewith clearly supports that one skilled in the art would accept the murine models as reasonably correlating to the condition in human.

The Examiner also believed that undue experimentation would be required to determine screening and testing protocols. However, as is apparent from the claim, the goal of the invention is to treat diabetes, i.e. achieve and maintain normoglycemia. Methods for determining whether normoglycemia is present have been exceedingly well known for many years; only routine blood glucose monitoring would be required to demonstrate the efficacy of the claimed invention.

5. Compliance with 35 U.S.C. 103(a)

The Examiner has rejected Claims 1-4 and 6-14 under 35 U.S.C. 103(a) as being unpatentable over US Patent 6,703,017 or by US Patent 5,425,764, or US Patent 5,629,194 each in view Posselt et al. (1991 *Ann. Surg.* **214**:363-373). Pursuant to MPEP 2143, in order to establish a *prima facie* case of obviousness three requirements must be met: First there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. In the case of the present invention, the cited references fail to suggest all of the claim limitations.

The '017 patent describes implanting insulin-producing cells in a dose of about 8,000-12,000 islets/kg of patient's body weight (col. 14, lines 7-9) to create a pancreas-like structure in a human patient. Therefore, the implant in USP '017 is designed to treat diabetes by creating a live "insulin pump" in the body. Furthermore, Example 12 of USP '017 describes implanting 5,000 islets per NOD mouse (this dose equals 200,000 islet/kg of body weight), which resulted in normoglycemia in these animals. The '017 patent does not teach or suggest implanting a dose of insulin-producing cells encapsulated in a biologically-compatible membrane prior to implanting the fully-therapeutic dose, wherein the tolerizing dose is at least one order of magnitude less than the therapeutic dose.

US Patent 5,425,764 describes a method of using an implantable bioartificial pancreas device containing insulin-secreting islets, to supply an exogenous source of insulin to treat the symptoms of diabetes. Accordingly, the '764 patent requires implantation of a therapeutic dose of insulin-secreting cells, i.e. the dose necessary to achieve normoglycemia. As such, the '764 Patent does not describe or suggest implanting a tolerizing dose of insulin-producing cells

encapsulated in a biologically-compatible membrane prior to implanting of fully therapeutic non-encapsulated dose, where the tolerizing dose is at least one order of magnitude less than the therapeutic dose.

US Patent 5,629,194 describes a method of implanting embryonic porcine pancreatic non-insulin-secreting cells capable of proliferating *in vivo* and then secreting insulin after transplantation. The dose sufficient for the treatment of insufficient insulin activity is 100,000-500,000 aggregates, each containing 300-500 cells per human patient. This is a fully therapeutic dose. Thus, the '194 patent does not describe or suggest implanting a tolerizing dose of insulin-producing cells encapsulated in a biologically-compatible membrane prior to implanting of fully therapeutic non-encapsulated dose, where the tolerizing dose is at least one order of magnitude less than the therapeutic dose.

Posselt et al. describes implanting unencapsulated islets into various areas of the body, liver, kidney, and thymus, of spontaneously diabetic BB rats. The only implantation site that showed survival of the implanted cells was the thymus. The intrathymic islet recipients were observed for a period close to the life span of the rat, without any recurrent diabetes. In additional experiments, approximately 100 days after the initial intrathymus transplantation, the transplanted rats were challenged with an extrathymic allogeneic islets, which remained intact even after removal of the thymus bearing the islet allografts. However, as the authors stated several times in this article, thymus is considered to be an immunologically privileged site and is subject to the usual biologic characteristics of such sites, in that prior sensitization of the host with skin allografts precludes prolonged survival of intrathymic islets (page 272, right column). The experiments, performed by Posselt et al. show just that, i.e. when allogeneic islets were transplanted into the thymus of recipients that had previously rejected donor strain skin grafts, the islets were destroyed in an accelerated manner, demonstrating that the intrathymic site is readily accessible to activated T cells (page 367 left column, and page 368, right column), and that no tolerance can be achieved using this protocol. Furthermore, Posselt et al. goes on stating that the achieved tolerization to intrathymic allografts is due to their direct influence on maturing thymocytes, which are more susceptible to tolerance-inducing signals, and that such "inappropriate" presentation of antigen by nonlymphoid cells induce a state of anergy in T cells (page 373). Therefore, Posselt et al. teaches away from using tolerizing dose of insulin-producing cells anywhere but thymus, and it does not teach encapsulating these cells.

Furthermore, as Dr. Scharp states in his Declaration submitted herewith, the BB rat has multitude of immunologic disorders that makes it more of a model for immune deficiency than for diabetes. Therefore, the BB rat is no longer considered an acceptable model for studying human autoimmune diabetes. This is also stated by Posselt et al.: "BB rats are known to be significantly immunodeficient" (see page 365, left column, line 5-6).

Here, the instant method is not limited to any specific implantation site for the tolerizing dose of encapsulated cells and still ensures survival of subsequently implanted un-encapsulated cells. Furthermore, opposite to Posselt et al., it works even in models where the immune system has already been sensitized.

Therefore, none of the cited references suggest the claimed limitation that a tolerizing dose is implanted prior to a therapeutic dose. Accordingly, even when combined, these references do not teach all the limitations of the claimed invention. As such, the cited references fail to support a *prima facie* case of obviousness. Therefore, Claims 1-4 and 6-14 are in compliance with 35 U.S.C. 103.

The Examiner has rejected Claim 5 under 35 U.S.C. 103(a) as being unpatentable over US Patent 6,703,017 or by US Patent 5,425,764, or US Patent 5,629,194 each in view Posselt et al. (1991 *Ann. Surg.* **214**:363-373) as applied to Claims 1-4 and 6-14, and further in view of USP 5,529,914. Non-obviousness of the independent Claim 1 in view of US Patent 6,703,017 or by US Patent 5,425,764, or US Patent 5,629,194 each in view Posselt et al. is asserted above. The US Patent 5,529,914 discloses a method of encapsulating cells, but it fails to cure the deficiencies of US Patent 6,703,017, US Patent 5,425,764, US Patent 5,629,194, and Posselt et al. Therefore, Claim 5 is in compliance with 35 USC §103(a).

CONCLUSION

Applicants have endeavored to address all of the Examiner's concerns as expressed in the outstanding Office Action. Accordingly, amendments to the claims, the reasons therefor, and arguments in support of the patentability of the pending claim set are presented above. In light of the above amendments and remarks, reconsideration and withdrawal of the outstanding rejections is specifically requested. If the Examiner finds any remaining impediment to the prompt allowance of these claims that could be clarified with a telephone conference, the Examiner is respectfully requested to initiate the same with the undersigned.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

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APPENDIX 1

Non-Obese Diabetic (NOD) Mouse BAC Library

The Wellcome Trust Sanger Institute (Cambridge, United Kingdom) recently released to the scientific community a Non-Obese Diabetic (NOD) mouse BAC library containing 240,000 clones obtained from the Diabetes and Inflammation Laboratory (Cambridge, United Kingdom).

The NOD mouse, which spontaneously develops type 1 diabetes, is a valuable animal model that is used extensively in research exploring the etiology, prevention, and treatment of this disease. It is a vital research tool for testing promising prevention and treatment strategies at the preclinical level.

The Sanger Institute sequenced the complete NOD BAC library and used this resource to complete a physical map of the BAC clones. As a next step, they plan to sequence the 200,000 clones from the Pieter de Jong library (Children's Hospital, Oakland CA). These sequences will be aligned with those from the most recent version of the normal C57B1/6 (B6) mouse strain (a non-diabetic mouse strain) in an effort to identify single nucleotide differences between NOD mouse clone end sequences and the B6 mouse genome. Investigators at the Sanger Institute will use this sequencing information to identify and map candidate genes. Such information will guide efforts to isolate genes that contribute to the development of type 1 diabetes in humans.

This research was conducted as part of the Immune Tolerance Network, which is jointly funded by the National Institute of Allergy and Infectious Diseases, the National Institute of Diabetes, Digestive, and Kidney Disorders (both part of the National Institutes of Health) and the Juvenile Diabetes Research Foundation.

Contents
NIAID Home | Search NIAID

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APPENDIX II

◆ REVIEW ARTICLE ◆

Taylor&Francis healthsciences

Development of new strategies to prevent type 1 diabetes: the role of animal models

Arno Hänninen[1], Emma Hamilton-Williams[2] and Christian Kurts[2,3]

Type 1 diabetes is an immune-mediated disease typically preceded by a long preclinical stage during which a growing number of islet-cell-specific autoantibodies appear in the serum. Although antigen-specific T lymphocytes and cytokines rather than these autoantibodies are the likely executors of β-cell-destruction, these autoantibodies reflect the existence of autoimmunity that targets islet β-cells. Abrogation of this autoimmunity during the preclinical stage would be the key to the prevention of type 1 diabetes. However, the quest of protecting islet-cells from the immune attack requires detailed knowledge of mechanisms that control islet-inflammation and β-cell-destruction, and of mechanisms that control immune tolerance to peripheral self-antigens in general. This knowledge can only be obtained through further innovative research in experimental animal models. In this review, we will first examine how research in non-obese diabetic mice has already led to promising new strategies of diabetes prevention now being tested in human clinical trials. Thereafter, we will discuss how recent advances in understanding the mechanisms that control immune response to peripheral self-antigens such as β-cell antigens may help to develop even more selective and effective strategies to prevent diabetes in the future.

Keywords: antigen-presentation; dendritic cells; disease prevention; immunological tolerance; non-obese diabetic mice; T-lymphocytes; transgenic animal models; type 1 diabetes

Ann Med 2003; 35: 546–563

Introduction

Type 1 diabetes (T1DM) is the clinical manifestation of the loss of insulin production in the endocrine pancreas. This is caused by immune-mediated destruction of islet β-cells. T1DM is believed to be an autoimmune disease, based on several lines of evidence (1–9). Although an infective (e.g., viral) or other environmental (e.g., dietary) agent may well be involved in the initiation of the immune attack towards islet β-cells (10, 11), it is clear that the (auto)immune attack itself is a key element in disease pathogenesis (12).

Immune responses against various islet-antigens appear even years before the clinical manifestation of T1DM, and precede the phase when sensitive metabolic tests first reveal attenuation in the function of β-cells (13). A number of islet-antigens are targeted by the immune system in T1DM, and cellular and/or humoral immune responses are detected against insulin, glutamic acid decarboxylase-65 (GAD 65), tyrosine phosphatase IA-2 and heat shock protein 60 (hsp 60) (14–16). Studies on pancreas samples obtained shortly after onset of clinical disease (as biopsies or as autopsy material following fatal ketoacidosis) have revealed the existence of cellular infiltrates in islets consisting of lymphocytes and antigen-presenting cells (APC), reflecting the immune attack and selective destruction of insulin-producing β-cells (4–6). Although studies in humans have been essential in the characterization of the disease process and its target antigens, development of novel therapeutic strategies requires studies also in experimental animal models.

The non-obese diabetic (NOD) mouse is the most widely used animal model of T1DM (17). This inbred mouse strain is unique in that it spontaneously develops autoimmune diabetes at high incidence. Although NOD mice harbour certain unique defects in their immune system (such as lack of the murine homolog of humal leukocyte antigen HLA-DR (18) and complement component C5 (19)) and have a

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Abbreviations and acronyms

APC	antigen-presenting cell
CTLA-4	cytotoxic T-lymphocyte antigen-4
DC	dendritic cells
GAD65	glutamic acid decarboxylase-65
GM-CSF	granulocyte-macrophage colony-stimulating factor
HLA	human leukocyte antigen
IA-2	islet-associated-2 (tyrosine phosphatase, an autoantigen)
IL	interleukin
IFN	interferon
hsp 60	heat shock protein 60
LCMV	lymphocytic choriomeningitis virus
MHC	major histocompatibility complex
NKT	natural killer cell-like T cell
NOD	non-obese diabetic mouse
OT-I T cell	OVA-specific CD8 T cell
OVA	ovalbumin
PD-L1	programmed death ligand 1
RANK	receptor for activation of NFκB
RIP	rat insulin promoter
TGF	transforming growth factor
T1DM	type 1 diabetes mellitus
TCR	T cell receptor
TNF	tumour necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
TRANCE	TNF-related activation induced cytokine
Treg	regulatory T cell

Key messages

- Type 1 diabetes (T1DM) is the clinical manifestation of immune-mediated destruction of insulin-producing β-cells which cannot be prevented yet.
- Most of the current clinical trials aiming at T1DM prevention are based on strategies developed in animal models.
- Research with these animal models has recently generated essential information regarding the cellular and molecular mechanisms regulating immune response to β-cells that can be used in the development of novel prevention strategies.

strong gender bias in diabetes incidence towards female preponderance (20), many of the important determinants are strikingly similar, including homologous susceptibility genes, major autoantigens, existence of insulitis and the ability of bone-marrow cells to transmit diabetes between individuals (21, 22).

In this review, we will first describe how research in non-obese diabetic mice has led to the discovery of new strategies of diabetes prevention that are now in human clinical trials. Thereafter, we will focus on work in transgenic animal models of islet-autoimmunity and discuss how immune tolerance towards sequestered self-antigens such as β-cell antigens is maintained, and how the tolerant state can change into a pathogenic immune response. Emphasis will be given to work elucidating the cellular and molecular basis of this balance and on therapeutic approaches that aim at posing this balance away from pathological autoimmunity.

The NOD mouse – a platform for testing existing immunomodulatory agents in the prevention of spontaneous autoimmune diabetes

Application of modern methods in molecular and cell biology to research on the mammalian immune system has allowed numerous receptor-ligand pairs and intracellular signalling pathways that regulate the function of lymphocytes and antigen-presenting cells to be identified. Through this research, an increasing number of antibodies and other reagents have become available for selective blockade or mimicking of the function of a specified molecule with a regulatory function. The NOD mouse is ideal for testing the effect on diabetes incidence of such agents. Thus, it forms a platform for identification and validation of potential drug targets and drugs in diabetes prevention.

In fact, surprisingly many different manoeuvres (over a hundred or so) have been reported to delay or diminish diabetes incidence in NOD mice (17). Many of these lack direct relevance for prevention of T1DM in humans simply because of their experimental nature. However, several potentially relevant approaches have been identified (Table 1 and references therein). As will be discussed later, some of the earliest human trials based on these approaches have now given negative outcomes that could reflect differences between T1DM in humans and the corresponding syndrome in NOD mice. However, results of other trials are more consistent with those obtained in NOD mice and support the idea that the NOD model can be used as a platform for testing various prevention protocols. Protocols applied in the NOD model have been based firstly on blocking the activation or function of T-lymphocytes or their subtypes and hence, of pathogenic effector cells, or their migration into pancreatic islets. Secondly, they have been based on administration of autoantigens in forms and schedules that are anticipated to enhance regulation of specific immune responses. Thirdly, numerous antigen-non-specific substances with immunomodulatory effects, or possible protective effect on β-cells have been applied with often favourable outcomes.

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It is conceivable that targeting a subtype of T-lymphocytes or a costimulatory pathway will affect the immune system also on a general level and induce various side effects. Therefore, there has long been a quest to develop strategies that selectively target the immune effector cells or locally inhibit tissue destruction. Accordingly, autoantigens (pro)insulin, GAD65 and hsp-60 or their peptides have been administered without immune adjuvants and/or *via* tolerogenic routes, especially intravenously or *via* mucosal surfaces (orally, intranasally or inhaled as aerosol). Results of these experiments have been promising (Table 1 and references therein) and have already led to human clinical trials, as detailed below. Unfortunately, initial results of these trials have been less encouraging (see below). According to animal experiments, oral and intranasal administration of antigen can also lead to induction of cytotoxic T-cell immunity (*53–55*) that may counteract its tolerance-promoting effects. This might need more attention in current trials where autoantigens are administered *via* mucosal routes. In animal models, a promising new strategy to induce regulatory immunity and protection from autoimmune diseases including diabetes is genetic (that is, DNA) vaccination with plasmids that encode an autoantigen (*56*). This strategy will be discussed later in this review.

Current clinical trials based on strategies developed in NOD mice

Strategies employed currently in clinical trials to prevent T1DM include the use of autoantigens in a tolerance-promoting form, targeting T-lymphocytes with antibody, protecting islet β-cells from the action of inflammatory cytokines and non-specific stimulation of regulatory cell types. Most of the ongoing trials, which are summarized in Table 2, are based on strategies developed in the NOD mouse. We will describe these trials shortly.

Anti-CD3 monoclonal antibody

hOKT3γ1[Ala-Ala] is a humanized non-activating anti-CD3 antibody engineered to lack Fc-Receptor binding domains (*57*). Preclinical studies in the NOD mouse showed that antibody treatment could effec-

Table 1. Examples of protocols that prevent diabetes in NOD mice and have a relevant target mechanism.

Protocol	Mechanism of action	Antigen specificity	Reference
Treatment with an antibody against:			
CD3	Depletion or inactivation of islet-infiltrating T cells/induction of regulatory immunity	no	(23, 24)
CD4/CD8	Depletion/inactivation of CD4 or CD8 T cells	no	(25)
TCR	Depletion/inactivation of T cells	no	(26)
CD86(B7.2)	Interference with T cell activation	no	(27)
CD45RB	Interference with T cell activation	no	(28)
CD40L	Interference with T cell activation	no	(29)
VLA-4 (α4-integrin)	Prevention of lymphocyte accumulation in islets and/or activation of diabetogenic T cells	no	(30, 31)
MAdCAM-1	Prevention of lymphocyte accumulation in islets and/or activation of diabetogenic T cells	no	(32)
ICAM-1/LFA-1	Prevention of lymphocyte accumulation in islets and/or activation of diabetogenic T cells	no	(33)
Treatment with:			
adjuvant (IFA)	immune deviation	no	(34)
vitamin D3 deriv.	immune deviation	no	(35)
αGal-ceramide	activation of regulatory NK T cells	no	(36)
interleukin 4	immune deviation/regulatory cells	no	(37)
interleukin-10	immune deviation/regulatory cells	no	(38, 39)
nicotinamide	protection of target β-cells against NO	no	(40)
subcutaneous insulin	Inactivation of pathogenic T cells	yes	(41, 42)
oral, intranasal or aerosol insulin	Induction of regulatory T cells/inactivation of pathogenic T cells	yes	(43–46)
insulin-encoding DNA-plasmid	Induction of regulatory T cells/inactivation of pathogenic T cells	yes	(47)
intranasal GAD peptides	Induction of regulatory T cells/inactivation of pathogenic T cells	yes	(48)
GAD-encoding DNA-plasmid	Induction of regulatory T cells/inactivation of pathogenic T cells	yes	(49, 50)
intrathymic GAD	Deletion of GAD-specific T cells from the pool of circulating mature T cells	yes	(51)
HSP 60	Inactivation of pathogenic T cells	yes	(52)
p277 of HSP 60	Inactivation of pathogenic T cells	yes	(52)

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Table 2. Currently ongoing clinical trials to prevent diabetes.

Trial	Principle/hypothesis	Evidence from NOD mice	Status	Results[a]
hOKT-3γ1	Inactivation of T cells. Induction of regulatory immunity	yes	Phase I-II	+
p277 of HSP-60	Induction of tolerance towards one autoantigen	yes	Phase I	+
insulin i.v. and s.c.[b]	Induction of tolerance towards one autoantigen	yes	Phase II	–
insulin s.c. in adjuvant (IFA)[c]	Induction of tolerance towards one autoantigen	yes	Phase II	N.A.
insulin *via* oral route	Induction of tolerance towards one autoantigen	yes	Phase II	N.A.
insulin *via* intranasal route	Induction of tolerance towards one autoantigen	yes	Phase III	N.A.
GAD 65 s.c.	Induction of tolerance towards one autoantigen	yes	Phase II	N.A.
nicotinamide *via* oral route	Protection of islet β-cells	yes	Phase III	–
IFN-α	Modulation of immune response	yes	Phase II	N.A.
Lactobacillus *via* oral route	Stimulation of regulatory NK-T cells	yes	Phase II	N.A.
Apl[d] of insulin B-chain	Induction of tolerance to insulin	yes	Phase II	N.A.
Vitamin D3 derivative	Modulation of immune response	yes	Phase II	N.A.
Exclusion of bovine proteins from diet in infancy	Avoidance of early immune response towards putative 'mimics' of autoantigens and/or sensitization of immature gut to foreign proteins	not directly	Phase III	N.A.

[a] Results: + positive, – negative results; N.A. results not available yet; [b] i.v. = intravenous administration; s.c. subcutaneous administration; [c] IFA = incomplete Freund's adjuvant; [d] *Apl* = altered peptide ligand.

tively 'cure' diabetic mice, restoring normoglycemia (24) or prevent disease development in pre-diabetic mice (58, 59). The antibody appears to bind to all CD3 expressing T-cells resulting in partial T cell receptor (TCR) signalling, which then has different outcomes depending on the cell type triggered. The overall outcome being killing or anergy induction in Th1 type cells (producing interleukin-2 or interferon-γ) and stimulation of Th2 type cells (cells producing interleukin-4 or interleukin-10) (60, 61). In an intervention trial, a 14 day course of intravenous hOKT3-γ1[Ala-Ala]) was used in recent onset (within 6 weeks) T1DM patients and followed for one year. Treatment resulted in sustained or improved C-peptide responses in 9 out of 12 treated patients compared with a sustained response in only 2 out of 12 control patients. Treated patients also needed significantly less insulin over the year following diagnosis. A transient depletion (36%) of lymphocytes followed antibody treatment, which later returned to normal levels. Responding patients had a decreased ratio of CD4:CD8 T cells after repopulation. Mild side effects included anti-idiotype antibodies, mild fever and an eczematous dermatitis-like rash. This trial is now being expanded to a multicentre phase II trial involving about 80 patients after these promising results. Further details are available at http://www.immunetolerance.org/research/autoimmune/trials/herold1.html

Hsp-60 peptide p277

One of the many self antigens, which are reacted against in T1DM, is heat shock protein 60. NOD mice contain autoreactive T cells specific for peptide 277 derived from this protein and immunisation of adult NOD mice with this peptide could prevent and occasionally revert diabetes (52). The mechanism of action is believed to be stimulation of Th2 type hsp60-reactive T cells resulting in a change in the cytokine milieu away from inflammatory cytokines (62). DiaPep277 is the human form of this peptide, which has been modified at two residues to increase stability *in vivo* (63). In a randomised double blind phase II trial DiaPep277 was injected subcutaneously in mannitol and vegetable oil at 0, 1 and 6 months following entry into the trial (63). Adult male patients were on average 12–15 weeks post-diagnosis and were followed for only 10 months. At the end of the study mean C-peptide levels had been maintained in treated patients whereas they had fallen in controls. Insulin requirements were also significantly lower at 10 months. Further phase II and phase III trials are now beginning based on these suggestive results.

Subcutaneous insulin therapy

Insulin has long been viewed as a primary target for tolerisation therapy in T1DM. It was shown that incidence of diabetes was significantly reduced in NOD mice given a low dose of prophylactic insulin from weaning until 180 days of age (41). Similar findings were also observed in the BioBreed rat, another animal model of T1DM (64). This phenomenon was believed to be due to 'beta-cell rest' in which there was a lower requirement for insulin secretion by the beta cells and less release of islet autoantigens associated with insulin secretion. These findings paved the way to the establishment of the Diabetes prevention trial (DPT-1) in which either subcutaneous or oral insulin was given to high risk relatives of T1DM patients. In the subcutaneous branch of the trial 84,228 relatives were assessed immunologically, metabolically and genetically for risk of disease

development over 5 years (DPT-T1D study group 2002). A group of 169 high risk (>50% over 5 years) relatives then underwent intervention of daily s.c. insulin injections and 170 controls received no treatment. The incidence of diabetes after 5 years was identical in both groups (69 *versus* 70 diabetic subjects). The reason for the negative outcome in the DPT-1 trial compared with the positive results in animal models and a pilot trial in humans (65) is unknown. It may be due to the dosage protocol or the difference in time of intervention. In mice, therapy began at a young age before the development of insulitis whereas relatives were selected on the basis of showing signs of autoimmunity (presence of islet autoantibodies) already.

Mucosal insulin

The second part of the DPT-1 trial is ongoing and used the 'intermediate' risk (25%–50% risk of developing T1DM over 5 years) relatives of T1DM patients. Patients receive continuous oral insulin or placebo and are being followed for 6 years for diabetes development. This intervention was not predicted to prevent diabetes by 'beta-cell rest' as subcutaneous insulin was but rather through active tolerisation upon antigen uptake at a mucosal surface (66). In NOD mice, an active (transferable), form of tolerance can be induced by feeding mice insulin but it is highly dose-dependent (43, 44). Therefore it is likely that in transferring this therapy to the human situation the dosage protocol will be critical.

A second major trial, the diabetes prediction and prevention project (DIPP), also seeks to tolerise individuals predicted to have a high risk of progression to T1DM by mucosal insulin administration. In this study babies are screened at birth for genetic HLA markers predisposing them to T1DM and then undergo immunological follow up. At risk children are then enrolled in a prevention trial to test intranasal insulin *versus* placebo for its ability to prevent or delay disease onset over three years. Aerosol insulin and intranasal insulin peptide administration have both been shown to be efficacious in prevention of diabetes in the NOD model (45, 46), although intranasal antigen administration has also been shown to induce potent cytotoxic T-cell immunity (67). For further details, see http://www.utu.fi/research/dipp/engdexx.htm

Subcutaneous insulin B chain in IFA

Another trial also attempts to induce tolerance to the metabolically inactive B chain of insulin by subcutaneous injection in Incomplete Freunds Adjuvant (IFA). In the NOD mouse, subcutaneous insulin or insulin B-chain given in IFA generated a transferable suppressive effect (42). In this study patients with insulin autoantibodies receive one injection within one month of diagnosis of T1DM and will be followed for two years. For further details, see http://www.immunetolerance.org/research/autoimmune/trials/orban1.html

Subcutaneous GAD65 in alum

An important early autoantigen in diabetes in both the NOD mouse and humans is GAD65 (51). A phase II trial will test the ability of recombinant GAD65 in alum to halt disease progression in recent onset patients. The vaccine called Diamyd will be injected subcutaneously two times four weeks apart in a range of doses. For further details, see http://www.diamyd.com/docs/research.html

Nicotinamide

A different approach, which is not antigen specific, attempts to use high doses of the B-vitamin nicotinamide to prevent or delay T1DM onset. Nicotinamide acts on the islet beta cells themselves making them more resistant to autoimmune attack. Nicotinamide is thought to primarily target the enzyme poly(ADP-ribose)polymerase (PARP) which is upregulated early after exposure to nitric oxide or reactive oxygen intermediates. Such exposure causes depletion of intracellular nicotinamide adenine dinucleotide (NAD^+) and results in death of sensitive cell types such as beta cells (68). Early nicotinamide treatment of NOD mice prevented diabetes partially and reduced insulitis severity (40). The results of initial clinical trials (69–71) have been controversial, since, while Chase et al. reported no effect of oral administration of nicotinamide, both Mendola and collaborators and Pozzilli et al. observed an increased stimulated C-peptide secretion in postpubertal patients on nicotinamide.

Nicotinamide has also been used in prevention trials with relatives of T1DM patients. One German study of 25 nicotinamide treated and 30 placebo control subjects showed no effect on disease progression (72). However, a larger study in New Zealand involving schoolchildren who had islet-cell autoantibodes obtained results that suggested a protective effect (73). The European nicotinamide diabetes intervention trial (ENDIT) is a larger clinical trial involving 552 high-risk relatives taking daily oral nicotinamide or placebo. For further details and for the recently disclosed negative results of this trial, see http://www.bris.ac.uk/Depts/DivMed/endit.html

Interferon-α

One successful strategy to prevent disease in the NOD

 ISSN 0785-3890

mouse involves altering the cytokine milieu to an anti-inflammatory, i.e., interleukin (IL)-4 and IL-10 containing environment. This has been done by injection of these cytokines, transgenic expression or by stimulation of cells which can produce them endogenously. Brod and co-workers have fed NOD mice with interferon-α, which suppressed diabetes and stimulated mitogen induced IL-4, IL-10 and interferon (IFN)-γ production in splenocytes (74). This was then trialled in 10 newly diagnosed T1DM patients resulting in preserved beta-cell function in 8 of these patients after 12 months (75). This study has now been expanded to a larger phase II trial involving 120 newly diagnosed patients taking oral IFN-α daily. It is unclear however what the exact mechanism of this therapy is, as interferon administered orally does not appear to be adsorbed into the bloodstream (76). Additionally, the presence of transgenically expressed IFN-α in the islet beta cells themselves actually precipitates diabetes (77). As such it is possible that interferon-α acts on lymphocytes in the gut, which then circulate to have a wider regulatory effect.

Oral administration of lactobacillus

Another study also attempts to modulate the immune response *via* the gut. Maclaren and co-workers are conducting a trial of oral lactobacillus in recently diagnosed T1DM patients. They propose that this will stimulate natural killer cell-like T (NKT) cell activity as administration of lactobacillus plantarum to children with HIV boosted this cell-subset (78). NKT cells are a regulatory-cell subset which have been reported to be deficient in T1DM patients (79, 80) although a more recent study utilising a more direct tetramer based method of identifying NKT cells refutes this claim (81). In the NOD mouse both transfer of this cell subset or injection of a glycolipid ligand to activate existing NKT cells can prevent diabetes (36, 82, 83). Lactobacillus casei feeding to NOD mice prevents disease (84) and various lactobacilli strains have been shown to be strong stimulators of IL-12 and IL-10 (85). However, strong evidence of lactobacillus stimulation of NKT cells has yet to be demonstrated.

Altered peptide ligand NBI-6024

An interesting antigen specific therapy involves the identification of peptides with similar sequences to immunodominant epitopes, which have modulatory activity. In the NOD mouse peptide 9-23 of the insulin B chain is recognised by a large proportion of pathogenic CD4 T-cells derived from the insulitic infiltrate (86–88). Alleva and colleagues searched for peptide analogues of $B_{(9-23)}$ which could inhibit $B_{(9-23)}$ specific T-cell responses and used one of these, NBI-6024, to test its therapeutic effect in the NOD mouse (89). Therapeutic altered peptide ligands are thought to function as competitive inhibitors of the native peptide by having a high binding affinity for MHC while concurrently engaging the TCR in a non-productive manner (90). They have also been shown to be effective in stimulating regulatory cell subsets, which can transfer protection between animals (91). The altered peptide ligand NBI-6024 is now being used in a large phase II study in which new onset T1DM patients will receive monthly injections of this medication at three different doses for two years.

1,25-Dihydroxy-Vitamin D3

The activated form of vitamin D3, 1,25-Dihydroxy-Vitamin D3, has been shown to act directly on the immune system *via* specific receptors on APCs and activated T cells (92). Its effects are immunosuppressive and include inhibition of IL-2 and IL-12 production and Th1-type responses (93). Importantly 1,25-Dihydroxy-Vitamin D3 has been shown to inhibit dendritic cell maturation, both *in vitro* and *in vivo* (94, 95). Administration of an analogue of 1,25-Dihydroxy-Vitamin D3 to NOD mice significantly reduced the incidence of diabetes (96) and enhanced the number of $CD4^+$ $CD25^+$ cells of regulatory phenotype in the pancreatic lymph node (97). 1,25-Dihydroxy-Vitamin D3 administered daily for 9 months in recently diagnosed T1DM patents is being trialled in a German study. For further details on this trial see http://www.roche.com/pages/downloads/science/pdf/rtdcmannh02-3.pdf

Withdrawal of cow's milk proteins from diet during infancy

According to one hypothesis (not directly derived from findings in NOD mice), exposure of the immature digestive tract in infancy to casein or other proteins in cow's milk formula or soy-based formula has a role in the early pathogenesis of type 1 diabetes. A casein-free diet has been tested in the NOD mouse after weaning and was shown to be effective in preventing diabetes (98). Although contradictory results were achieved from previous studies in humans evaluating whether introduction of formula during infancy is associated with the development of type 1 diabetes later in life (99, 100), a larger phase III clinical trial called TRIGR (Trial to Reduce IDDM in the Genetically at Risk) has been initiated to test if the hypothesis holds true or not. Infants that are determined to have a high risk of developing type 1 diabetes are eligible. After weaning from breast-feeding, they will receive hydrolyzed formula that does not contain intact proteins, or standard cow's milk-based formula. Infants will have at least a two-

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month exposure to the study formulae and then will be monitored for up to ten years. For further details, see http://www.trigr.org/about.html

Transgenic mouse models in the study of T cell-mediated autoimmunity against pancreatic islets

Investigations in humans with T1DM and studies performed in the NOD mouse model have clearly demonstrated the crucial role of autoreactive CD4 helper and CD8 effector T cells in the pathogenesis of autoimmune diabetes (101). In NOD mice, numerous autoantigens are recognized by these T cells (see above). The relevance of these antigens for disease initiation and progression, and the mechanisms of their processing and presentation, however, are still unclear. Consequently, techniques and tools other than the NOD model had to be developed. The most valuable models were generated by genetically introducing well characterized model antigens into islet-β-cells, under the assumption that such neoantigens will be handled like endogenous islet antigens by the immune system (102). Transgenic neoantigens, however, are often aberrantly expressed in the thymus, resulting in the deletion of endogenous transgene-specific T cells. This problem could be overcome by intravenous injection of specific T cells, which are usually obtained from congenic T cell receptor – transgenic mice. Since their antigen (the model autoantigen) is not expressed in the donor mice these T cells are not negatively selected in the thymus. Importantly, the absence of antigen during T cell development ensures a completely naïve phenotype of the autoreactive T cells. This is crucial because it allowed, for the first time, investigation of the activation of T cells specific for a pancreatic self antigen *in vivo* (103). The other major advantage of adoptive transfer experiments is the ability to label the T cells with fluorescent dyes prior to injection, which allows them to be easily tracked *in vivo*. This technique can also reveal their activation and proliferation through analysis of cell division (104, 105). These innovations allowed many important aspects of T cell-mediated autoimmune responses to be addressed, including the site of activation of the autoreactive T cells, the influence of their numbers, their fate and the type of antigen-presenting cell responsible. Although parameters such as T-cell affinity and epitope spreading and recruitment of other autoreactive T cell clones are not taken into account, this approach has added considerably to our knowledge of immune-mediated destruction of islet β-cells by allowing us to dissect the actions of relevant components and their interactions which finally result in disease (102).

The approach described above has been particularly successful in elucidating the role and physiology of CD8 T effector cells. As compared to CD4 T helper cells, CD8 T cells are difficult to maintain *in vitro* as T cell lines, mainly because of their cytotoxicity. They quickly destroy APCs *in vitro*, and thus deprive themselves of antigenic survival signals. Furthermore, the MHC class I molecules, which are recognized by CD8 T cells, are expressed by nearly all murine cells, including T cells, so that CD8 T cells could potentially kill each other *in vitro*. These problems were overcome by generating naïve, and therefore unarmed CD8 T cells in transgenic donor mice. Such T cells became cytotoxic only after transfer into recipient mice expressing antigen. Transgenic CD8 T cells specific for certain MHC class I molecules were first generated (106). These T cells were introduced into transgenic mice expressing MHC I molecules under the influence of the rat insulin promoter (RIP) in pancreatic islet cells. This resulted in immune tolerance, unless the T cells were supplied with inflammatory mediators (107). These experiments demonstrated that autoreactive CD8 T cells can in principle destroy β-cells and cause diabetes (102) although their specificity for antigenic peptide was unclear. Therefore, they did not allow investigation of the processing of islet antigens. This limitation was overcome in the next generation of CD8 T cell transgenic systems. The first of these models, the RIP-mOVA model, expressed ovalbumin (OVA) as a model antigen in pancreatic islet cells. OVA-derived peptides could be detected with transgenic OVA-specific CD8 and CD4 T cells derived from OT-I and OT-II mice, respectively (OT-I and OT-II refer to the transgenic OVA-specific T cells, called OT-I and OT-II cells, that are produced by these transgenic mice). These experimental systems demonstrated clearly that both naïve CD4 and CD8 T cells with specificity for a pancreatic self antigen are activated by dendritic cells in the pancreatic lymph nodes, and not in the islets (108) (Fig 1). The DCs presumably took up β-cell antigens in the islets and carried them to the pancreatic lymph node for presentation to naïve T cells. In the case of CD8 T cells, these results demonstrated so-called cross-presentation, which denotes the presentation of extracellular antigens with MHC I molecules to CD8 T cells.

Since then, numerous observations of cross-presentation and cross-priming *in vivo* have been described, not only of transgenic self-antigens in models like the RIP-mOVA or the hemagglutinin system (109), but also of tumour (110) and viral antigens (111, 112). Thus, the basic mechanisms of antigen presentation and T-cell activation *in vivo* that were uncovered in transgenic systems have been verified for many 'natural' antigens. This is not surprising, because indirect presentation of antigen

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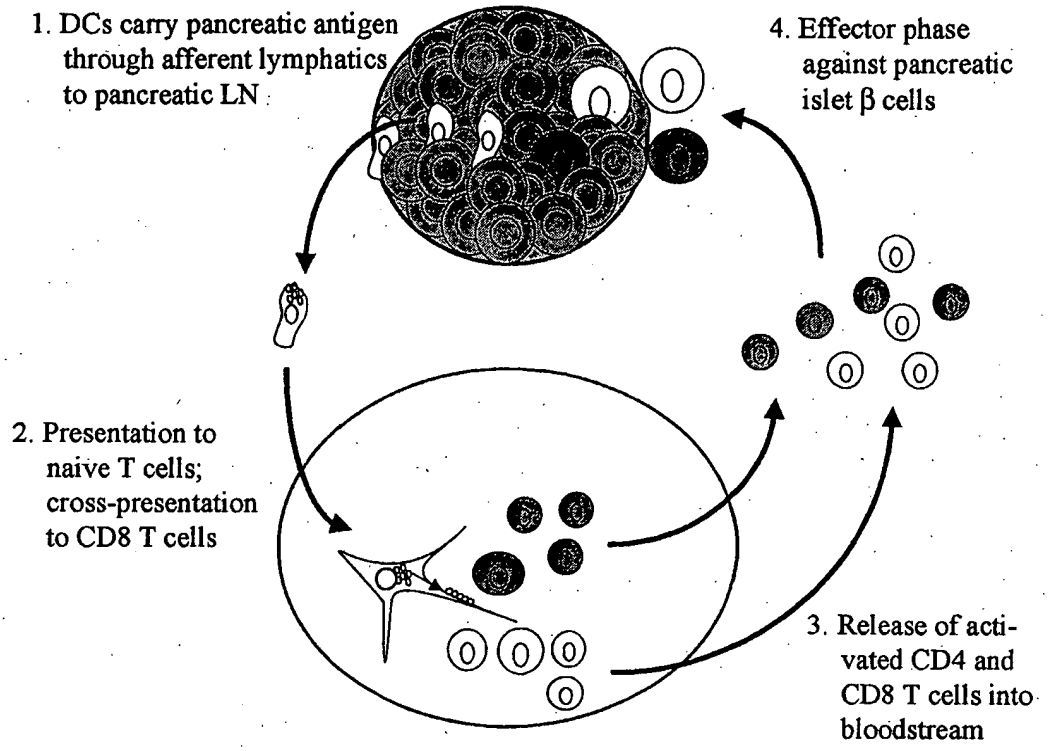

Figure 1. Antigen capture by dendritic cells in islets and their migration to the pancreatic lymph node for presentation of β-cell antigens to T lymphocytes. Antigen-presentation *via* both MHC II and MHC I pathways enables both CD4 and CD8 T cells to be activated. Activated T cells can enter non-lymphoid tissues such as pancreatic islets in search for antigen-expressing target cells (see text for details).

to T cells – both to CD4 and/or CD8 T cells – offers advantages to the immune system in the battle against infections antigens. Only indirect antigen presentation (in the case of CD8 T cells, cross-presentation) allows an immune response to be mounted against viruses that functionally compromise, or simply avoid antigen-presenting cells (103).

These basic mechanisms of antigen presentation, which were uncovered in model systems such as the RIP-mOVA system, were found to be important also for the pathogenesis of diabetes in NOD mice, in which CD8 T cells have emerged as important mediators of disease (113–117). TCR-transgenic NOD mice have been generated, and in many of these, the CD8 T cells destroyed pancreatic islets, whereas the CD4 T cells played more or less relevant helper roles (115, 118, 119). In NOD mice, autoreactive CD4 T cells appear to play an important role in diabetogenesis, and also their activation occurs in the pancreatic lymph nodes (120). Interestingly, presentation of autoantigen to autoreactive CD4 and CD8 T cells is age-dependent. It did not occur in very young animals (120), suggesting that developmental changes in the pancreatic tissue cause the release of autoantigens to the pancreatic lymph node. In this respect, a wave of physiological apoptotic β-cell death, which occurs in rodents at 14–17 days after birth (121), and in humans at birth (122), may be relevant. During such a wave, autoantigens are released from apoptotic islet cells and may activate autoreactive T cells with specificity for β-cell antigens (101, 123). Antigens released from apoptotic cells are taken up particularly well by dendritic cells (124–126) and normally, induce T cell tolerance (127). Why antigen-presentation results in autoimmunity in some individuals is not known, but inflammatory signals caused by accompanying infections (128), differences in the T cell repertoire, genetic susceptibility differences and dysregulated cellular death may be involved. Also, unphysiological β-cell death might cause the release of islet antigens in an immunogenic setting, and induce autoimmunity rather than tolerance (101). Tissue remodelling and replacement of β-cells occur also later. In the RIP-mOVA system, pancreatic tissue antigens were observed to be constantly shuttled to the draining lymph nodes of adult animals, where they were presented to autoreactive T cells (108, 120). These T cells indeed caused diabetes, but only when their precursor frequency was unphysiologically high (103). Physiological numbers of T cells were tolerized

 ISSN 0785-3890

by deletion before they could destroy all β-cells. Thus, cross-presentation of pancreatic self-antigen led to deletional CD8 T-cell tolerance, which was termed cross-tolerance (129). Cross-tolerance has also been shown in other transgenic systems, and its mechanisms have been elucidated (109). However, in some systems, most notably in those examining antigens derived from the lymphochoriomeningitis virus (LCMV), specific CD8 T cells ignored this self antigen (130) but were not deleted. One possible explanation is that various antigens are handled differently by the immune system. In this case, however, pathogens might easily evolve strategies to escape immune surveillance. An alternative explanation came from studies investigating the influence of antigen dose on cross-presentation. Only high antigen levels were cross-presented and induced cross-tolerance (131–133) while low dose self antigens were ignored, in which case the immune system must rely on ignorance to avoid autoimmunity. Thus, the level of antigen expression appears to determine the mechanism by which CD8 T-cell mediated autoimmunity is avoided.

Ignorance of self antigen allows autoreactive T cells to survive within the T cell repertoire. Such T cells could theoretically unleash their destructive potential if they were activated by other means, for example by pathogens that are similar in antigenic structure. This sword of Damocles has first been demonstrated in the LCMV system, where mice expressing determinants of the LCMV virus in pancreatic islets became diabetic after infection with the virus (134–135). Such a mechanism may explain why immune diseases are often observed after viral infections. Direct evidence for antigenic mimicry as a mechanism of diabetes induction, however, is scarce (136). In contrast to ignorance or to the induction of anergy or TCR downregulation in autoreactive T cells (137, 138) deletion of autoreactive T cells removes the threat of autoimmunity permanently by eliminating potentially harmful effectors. However, this mechanism can also fail, for example if the precursor frequency of the autoreactive CD8 T cells is too high, or when autoreactive CD4 T cells are present (103). Transgenic CD4 T cells specific for islet self-antigens appear to be less efficient as direct mediators of diabetes than CD8 T cells (139, 140). However, they act by delaying the deletion of CD8 effector T cells (139), by mediating their entrance into pancreatic islets (115) or by supporting their effector phase in the islets (107). But also the CD4 T cells themselves are subject to peripheral tolerance. CD4 T cells can be deleted (141) or functionally silenced (137). Also Th1 → Th2 diversion may happen (142) and regulatory $CD25^+$ CD4 T cells, that actively suppress immune responses (143) can be generated.

Molecular mechanisms regulating immune response to islet-antigens

As described above, antigens derived from islet-β-cells are under continuous surveillance by the immune system. Whether this antigen-presentation leads to expansion of islet-specific T lymphocytes and to the development of anti-islet immunity (Fig 1) – or to their silencing – is of crucial importance. Molecular mechanisms underlying this distinction are gradually becoming unravelled. Signalling via cytokine- and costimulatory receptors is of particular importance (144–147). For example, expression of the costimulatory ligand B7.1 or TNF-α on islets, and signalling between CD40L and CD40 can lead to the breakdown of the tolerant state to islet-antigens, as has been shown in NOD mice by antibody treatments and by expression of TNF-α or B7.1 on β-cells (148–150). The action of TNF-α depends, however, on the timing of its action and can also suppress β-cell destruction (149, 150).

The TNF- and TNF-receptor families include several members that may still turn out to be important in the regulation of islet-immunity. A good example is the demonstration that signalling through the TRANCE-RANK receptor-ligand pair is involved in the generation of CD25 + CD4+ regulatory T cells in the pancreatic lymph node (151). These cells were shown to be able to restrain diabetogenic CD8 effector T cells. The existence of regulatory T cells seems to be controlled by costimulatory molecules, because in the NOD mouse, lack of B7-2 or CD28 leads to acceleration of diabetes *via* impaired action of $CD25^+$ CD4 regulatory T cells (152).

Functional silencing, that is 'anergy', is traditionally considered to result from TCR-ligation without simultaneous costimulation but may in fact require costimulation. Accordingly, CTLA-4 is an important negative regulator of the activity of T cells (153), and abrogation of its function leads to acceleration of diabetes in BDC2.5 NOD mice (153). Also regulatory cells including $CD25^+$ CD4 cells (143) and Treg1 cells (154), are partly anergic themselves. Cytokines IL-10 and transforming growth factor (TGF) -β, produced by regulatory T cells, are important in peripheral tolerance via their inhibitory actions on dendritic cell activity and on T cell proliferation and differentiation (155, 156). Which role these regulatory cells play in human disease is not yet clear but is under intensive research.

Receptors that recognize pathogen-associated molecular patterns (PAMP) and are thus called pattern recognition receptors (PRR) are also important in the generation of immune responses (157). These receptors are expressed on cells of the innate immune system including dendritic cells and couple with intracellular signal transduction pathways that reg-

 ISSN 0785-3890

ulate gene expression. Binding of a ligand (i.e., a foreign pathogen) to such receptors can thus upregulate various functions of dendritic cells. Although it is quite unclear if PRR have any role in the pathophysiology of islet-specific autoimmunity, these receptors and their signalling pathways deserve attention when attempting to manipulate antigen presentation in pancreatic lymph node.

Consequently, costimulatory and/or death-receptors and cytokines expressed by the cells of our immune system form an intricate network of interacting receptors and ligands that influence the fate of islet-reactive T lymphocytes. This network contains a multitude of potential drug targets for attempts to restore tolerance to peripheral tissue antigens such as islet-antigens, some of which are depicted in Figure 2.

Selective modulation of immune response to islet-antigens

Restoration of tolerance to islet β-cells without compromizing general immune function requires that immunomodulation selectively targets the immune response to β-cells. So far, tolerance has been induced experimentally by introducing the antigen in a controlled fashion, i.e., without adjuvants, as a soluble protein and preferably *via* mucosal surfaces or the intravenous route (158, 159). As discussed above, the prospects of these manipulations as immune therapy to diabetes, however, remain very uncertain. Tolerance can also be induced in T cells specific for any given antigen by complexing antigenic peptides with MHC molecules to produce 'custom-made' T cell receptor ligands that are administered without adjuvants and thus in the absence of costimulatory signals (160, 161). This, however, requires detailed knowledge of the immunodominant epitopes of the antigen, and when effective, is likely to be strictly restricted to a narrow specificity of T cells that may not alone be responsible for disease pathogenesis. Thus, multiple T cell receptor ligands would need to be administered unless some of them would be able to induce active regulatory immunity (161).

Current knowledge of the cellular and molecular mechanisms underlying regulation of peripheral tolerance to islet-antigens suggests that new strategies of therapeutic manipulation of the detrimental islet-specific immune response await to be discovered. This would, however, require novel ways of introducing islet-antigens to the immune system in a way that would allow a tolerance-regulating mechanism to be

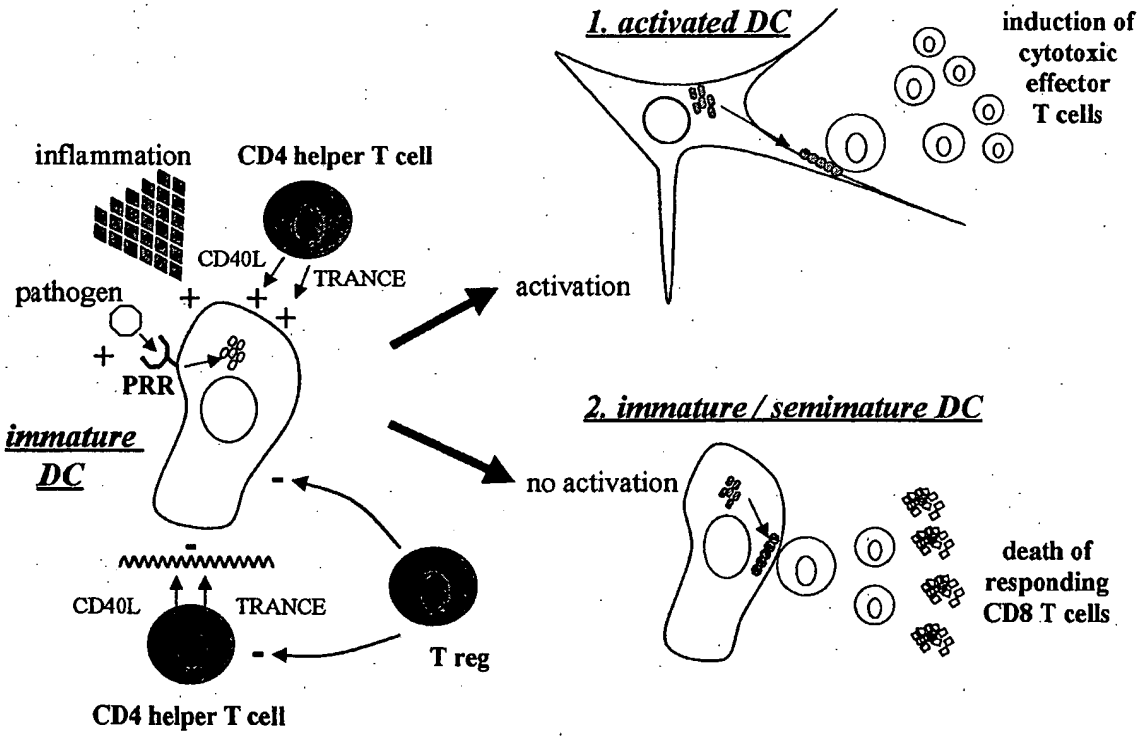

Figure 2. Factors that promote the activation of immature dendritic cell (DC) to become a potent antigen-presenting cell (1. activated DC) after endocytosis of antigen; and the role of regulatory T cells (Treg, e.g., CD25 + CD4 T cells) as opponents of the activation of immature DC (2. immature/semimature DC). PRR = pattern recognition receptor.

 ISSN 0785-3890

introduced simultaneously with the antigen in a strictly localized manner. Because T cells recognize antigen only when appropriately presented to them, this is possible only during T-cell interaction with an antigen-presenting cell or a target cell. In type 1 diabetes, the target cells (i.e., islet-β cells) are obviously beyond selective manipulation unless xeno-transplantation of pig islets derived from transgenic pigs (whose islets would be made to express immunomodulatory agents) is considered relevant as a treatment option. Interaction with an APC which is presenting an autoantigen is the other antigen-specific interaction that a diabetogenic T cell commits itself to. Therefore, immunomodulation should probably be targeted here. If this APC cell were made to express an immunomodulatory agent, it could perhaps selectively target this agent to the T cell that is engaged with it *via* its antigen-specific receptor (162). This might re-direct the T cell to a less aggressive behaviour instead of its expansion and maturation into a clone of islet-destructive T cells. The immunomodulatory agent could be a death-inducing ligand such as FasL, TRAIL, TNF or PD-L1 (163–165). Experimental evidence suggests that expression of FasL on dendritic cells renders them tolerogenic (166, 167) although this may not always happen (168). In fact, we have observed that ligation of Fas during T cell contact with dendritic cells costimulates a fraction of the responding T cells (169) which could complicate its effects as a death-ligand. For other death-receptor ligands, data do not yet exist.

Alternatively, the immunomodulatory agent could be a cytokine such as IL-10 or TGF-β, because these cytokines regulate the costimulatory activity of the antigen-presenting cell and proliferation and polarity of the responding T cell as discussed above (155, 156). For therapeutic purposes, this type of targeted immunomodulation could be achieved by *in vitro*-treatment of autologous antigen-presenting cells with the immunomodulatory agent together with the antigen before re-injection, or by genetic modification to make these cells express the immunomodulatory agent themselves. Also, although B lymphocytes as well as monocytes can present antigen to T cells, dendritic cells would likely suit best to be used as modified antigen-presenting cells. This is because only dendritic cells are able to induce a response both in naïve and antigen-experienced T cells (170), and because B lymphocytes have an intrinsic property of directing islet-reactive T cells into diabetogenic behaviour at least in the NOD mouse (171, 172).

Modification of antigen-presentation from dendritic cells – a therapeutic option?

Dendritic cells can be propagated from peripheral blood of humans with the aid of cytokines granulocyte macrophage colony stimulating factor (GM-CSF) and IL-4 (173, 174). Such dendritic cells could be treated *in vitro* with an immunomodulatory agent or perhaps transfected with a gene construct to make them express the immunomodulatory agent themselves and pulsed with antigen before re-injection back into the same individual. *In vitro* cultured and antigen-pulsed dendritic cells from healthy individuals can, in fact, either induce a potent immune response or tolerance depending on their prior *in vitro*-treatment when re-injected (175, 176). The limitations of this approach would come from the amount of work and potential safety risks introduced by *in vitro* propagation of autologous cells. These limitations would not apply to the use of purified DNA as a 'vaccine' (177). The discovery that injecting 'naked' DNA can induce immunity was made a decade ago (178), and vaccination with 'naked' DNA represents a promising strategy for inducing cell-mediated immune responses (including cytotoxic CD8 T cells) (177, 178). Purified plasmid DNA encoding both an autoantigen and an immunomodulatory agent could therefore work as a 'vaccine' that might elicit a modified immune response resulting in tolerance instead of effective cell-mediated immunity. Unlike cells that need to be autologous, a vaccine consisting of recombinant DNA once validated could potentially be applied to individuals of a diverse genetic background (i.e., irrespective of MHC haplotypes or other disparate features). Thus, a 'vaccine' for people at risk of developing T1DM could perhaps become available on a large scale. The safety risks of introducing foreign recombinant DNA would probably be small if vectors that are able to incorporate into host genome (i.e., retroviral vectors) would not be applied.

In the NOD mouse, a few studies already exist in which DNA-plasmids encoding insulin, its B chain, GAD or hsp 60 either alone or together with a plasmid encoding a regulatory cytokine (e.g., IL-4) have been injected as a 'vaccine' to induce tolerance (47, 179–181). DNA injected as a 'vaccine' is taken up by cells in the tissue including dendritic cells which express the antigen and present it to T cells (182). In many cases this has resulted in regulatory immunity and protection from disease. This is noteworthy, given the capacity of DNA-vaccination to induce effective cytotoxic T-cell immunity in tumour and viral disease models (183, 184). Hence, the control of possible adverse immune reactions deserves careful attention when using this strategy. We believe that simultaneous expression of the autoantigen with an immunomodulatory agent could be a possible way to better control such adverse reactions and to target immunomodulation most effectively to pathogenic T cells. Intensive research is

 ISSN 0785-3890

yet needed in models such as the RIPmOVA -model and the NOD mouse to identify the most effective immunomodulatory molecules and the most relevant islet-autoantigens that these should be combined with.

A promising strategy to induce tolerance towards a defined antigen is to make a fusion protein consisting of the antigen and an antibody to an endocytosis receptor expressed on immature dendritic cells. Accordingly, the model antigen ovalbumin when conjugated to an antibody against the DEC-205 receptor, induced antigen presentation in dendritic cells that remained in an immature state (185). Presentation of antigen *via* immature dendritic cells rendered mice tolerant to subsequent challenge with the same antigen showing that targeting the antigen thoughtfully to the immune system may elicit antigen-specific tolerance.

We have discussed here the role of animal models in development of therapeutic strategies to induce immune tolerance to islet-autoantigens and envisioned some novel approaches to modify antigen-presentation from dendritic cells to induce tolerance (Fig 3). These strategies and approaches are currently under investigation in many laboratories including our own laboratories. Via innovative and careful work in animal models of islet-autoimmunity some of these approaches may be transformed into strategies that could be applied as specific immunotherapy to type 1 diabetes. Compared to treatment with general immunomodulatory or immunosuppressive agents, or other general, although certainly effective, treatment options like bone marrow reconstitution (186), tolerance-promoting presentation of autoantigens to the immune system would offer a worthwhile choice.

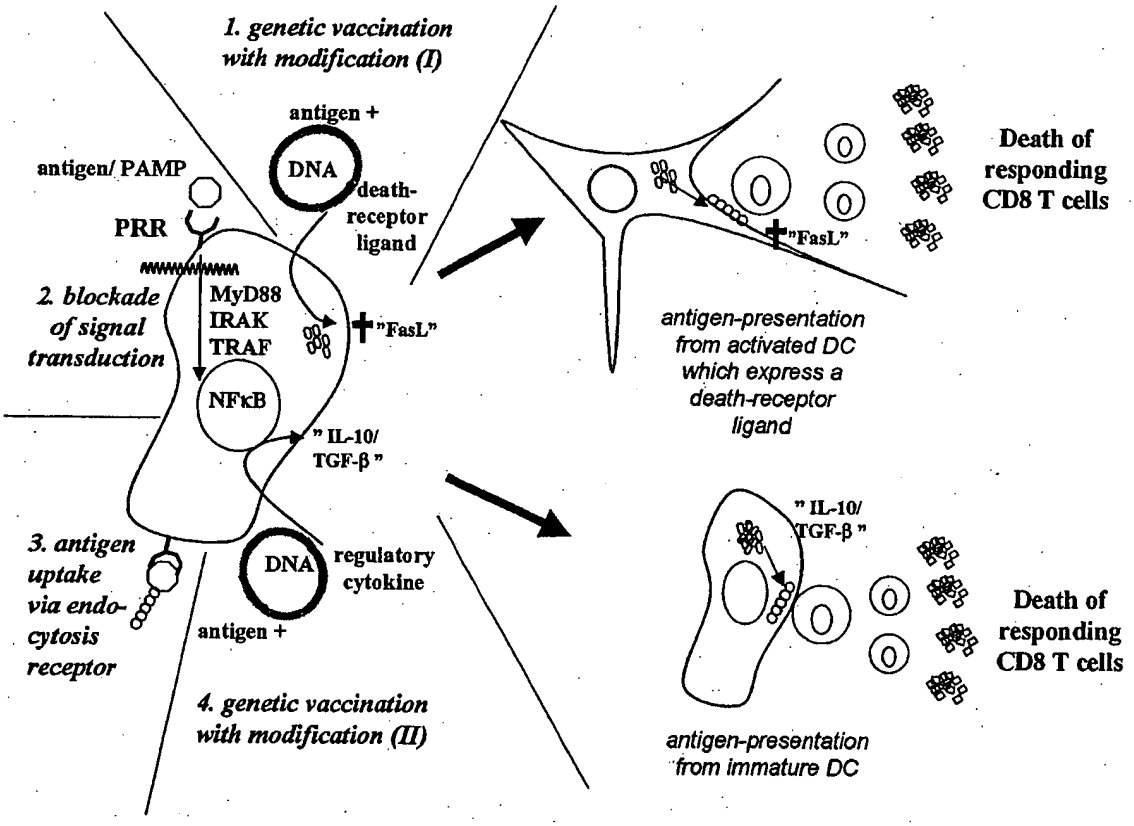

Figure 3. Four potential strategies for manipulating antigen-presentation in dendritic cells to induce tolerance. 1. Genetic (i.e. DNA) vaccination with a plasmid that encodes antigen and a death-receptor ligand (FasL, TRAIL, PD-L1/2); 2. Blockade of signal transduction from pathogen recognition receptor (PRR) by e.g., genetic modification of DC or antisense oligonucleotides; 3. Conjugation of antigen to a structure that binds to an endocytosis receptor (e.g., DEC-205) expressed on immature DC; 4. Genetic (i.e., DNA) vaccination with a plasmid that induces production of a regulatory cytokine (TGF-β, IL-10).

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/823,263	04/13/2004	Paul P. Latta	LATTA.002C4	3489

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EXAMINER	
BELYAVSKYI, MICHAIL A	

ART UNIT	PAPER NUMBER
1644	

DATE MAILED: 06/16/2005

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary

Application No.	Applicant(s)	
10/823,263	LATTA, PAUL P.	
Examiner	**Art Unit**	
Michail A. Belyavskyi	1644	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *09 May 2005*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-14* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-14* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1)☐ Notice of References Cited (PTO-892)
2)☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3)☒ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____.
4)☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5)☐ Notice of Informal Patent Application (PTO-152)
6)☐ Other: _____.

RESPONSE TO APPLICANT'S AMENDMENT

1. Applicant's amendment, filed 05/09/05 is acknowledged.

Claims 1-14 are pending.

Claims 1-14 are under consideration in the instant application.

Applicant's amendment filed 05/09/05 in conjunction with Declaration of Dr. Scharp under 37 C.F.R 1.132 demonstrating the effectiveness of implantation of a sub-therapeutic, tolerizing dose of insulin-secreting cells and further administering an therapeutic dose of insulin-secreting cells to treat diabetes, has obviated the previous enablement rejection under 35 U.S.C. 112, first paragraph.

In view of the amendment, filed 05/09/05 the following rejections remain:

2. The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

3. Claims 1-4 and 6 -14 stand rejected under 35 U.S.C. 103(a) as being unpatentable over US Patent 6,703,017 or by US Patent 5,425764 or US Patent 5,629,194 each in view Posselt et al (Diabetes, 1992, v.41, pages 771-775) for the same reasons set forth in the previous Office Action, mailed on 01/26/05.

Applicant's arguments, filed 05/09/05 have been fully considered, but have not been found convincing .

Applicant asserts that: (i) US Patent '017 requires implanting only therapeutic dose of insulin-secreting cells and does not suggest implanting a tolerizing doze of insulin-producing cell prior to implanting of fully therapeutic dose; (ii) US Patent '194 does not describe or suggest implanting of a tolerizing dose of insulin producing cells prior to implanting of fully therapeutic dose; (iii) Posselt et al., teaches away from using tolerizing dose of insulin producing cells anywhere but thymus.

Applicants have traversed the primary and the secondary references pointing to the differences between the claims and the disclosure in each reference. Applicant is respectfully reminded that the rejection is under 35 USC103 and that unobviousness cannot be established by attacking the references individually when the rejection is based on the combination of the references. see In re Keller, 642 F.2d 4B, 208 USPQ 871, 882 (CCPA 1981) See MPEP 2145. This applicant has not done, but rather argues the references individually and not their combination. One cannot show non-obviousness by attacking references individually where the rejections are based on a combination of references. In re Young 403 F.2d 759, 150 USPQ 725 (CCPA 1968).

As has been stated previously, US Patent '017 teaches a method of treating diabetes in a mammal comprising implanting insulin-producing cells encapsulated in a biologically compatible membrane (see entire document, Abstract and columns 6, 8, 9 –14 and Example 12 in particular) . US Patent '017 teaches that insulin producing cells are pancreatic islet cells from primary cell source (see columns 8 and 11 in particular). US Patent '017 teaches that pancreatic islet cells are from the same species as the mammal and are implanted interperitoneally into the tissue of a mammal beneath the kidney capsule (see overlapping columns 13-14 and Example 2 in particular). US Patent '017 teaches that encapsulation of said insulin-producing cells in biologically compatible membrane for success of implantation is well known in the art (see column 12 and Example 12 in particular).

US Patent '764 teaches a method of treating diabetes in a mammal comprising implanting insulin-producing cells encapsulated in a biologically compatible membrane (see entire document, Abstract and overlapping columns 5-6 in particular). US Patent '764 teaches that insulin producing cells are pancreatic islet cells (see column 1 and 4 in particular). US Patent '764 teaches that cells are implanted interperitoneally (see column 5 in particular).

US Patent '194 teaches a method of treating diabetes in mammal comprising implanting insulin-producing cells encapsulated in a biologically compatible membrane (see entire document, Abstract overlapping columns 7-8 , 12 and Example II in particular). US Patent '764 teaches that insulin producing cells are pancreatic islet cells (see column 8 in particular). US Patent '764 teaches that cells are implanted intaportal (see column 7 in particular). US Patent '194 teaches administration of one or more anti-inflammatory agent at the dosage sufficient to achieve the desired therapeutic effect. US Patent '194 teaches that said agent can be administered prior to at the same time or subsequent to administration of insulin-producing cells (see overlapping columns 14-15 in particular).

US Patent '017 or US Patent ' 764 or US Patent '194 does not explicitly teaches a method of treating diabetes in a mammal comprising administration two doses of insulin-secreting cells one tolerizing and one curative wherein tolerizing doze is one order less than curative.

With regards to the issue that Posselt et al., teaches away from using tolerizing dose of insulin producing cells anywhere but thymus. The Examiner disagrees with Applicant's interpretation of Posselt et al. Moreover, it is noted that the instant claims does not recited any specific place where a tolerizing dose of insulin-secreting cells should be implanting. It is the Examiner position that Posselet et al., teach that the important goal in the treatment of insulin-dependent diabetes by pancreatic islet transplantation is the development of strategies that allow permanent survival of pancreatic islet without continuous host immunosuppression. Posselet et al., further teach a strategy comprising two step process : first administering a small dose of cells that induces an unresponsive state, i.e. tolerizing dose and then administering fully therapeutic dose, at another site (see entire document, Abstract in particular). Posselet et al., teach that said strategy permits the survival of pancreatic islet transplant.

It would have been obvious to a person of ordinary skill in the art at the time the invention was made to apply the teaching of Posselt at al. to those of US Patent '017 or US Patent ' 764 or US Patent '194 to obtain a claimed method of treating diabetes in a mammal comprising administration two doses of insulin-secreting cells one tolerizing and one curative wherein tolerizing doze is one order less than curative

One of ordinary skill in the art at the time the invention was made would have been motivated to do so, because a strategy comprising two step process : first administering a small dose of cells that induces an unresponsive state, i.e. tolerizing dose and then administering fully therapeutic dose, at another site permits the survival of pancreatic islet transplant as taught by Posselet et al. Said strategy can used in the method of treating diabetes in a mammal, comprising implanting pancreatic islet, taught by US Patent '017 or US Patent ' 764 or US Patent '194. The strongest rationale for combining references is a recognition, expressly or impliedly in the prior art or drawn from a convincing line of reasoning based on established scientific principles or legal precedent, that some advantage or expected beneficial result would have been produced by their combination. In re Semaker. 217 USPQ 1, 5 - 6 (Fed. Cir. 1983). See MPEP 2144.

From the combined teaching of the references, it is apparent that one of ordinary skill in the art would have had a reasonable expectation of success in producing the claimed invention. Therefore, the invention as a whole was *prima facie* obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references, especially in the absence of evidence to the contrary.

Claims 8-14 are included because it would be conventional and within the skill of the art to : (i) determine the proper pore size for the permselective membrane or (ii) to determine the optimum dosage and means of administration of insulin-secreting cells in an absent of a showing of unobvious property. Moreover, Applicant acknowledge that one of ordinary skill in the art can readily determine the proper pore size for the permselective membrane (see page 8, line 13-20

of the instant Specification in particular). Further, it has been held that where the general conditions of a claim are disclosed in the prior art, discovering the optimum or workable ranges or means of administration involves only routine skill in the art. *In re Aller*, 220 F2d 454,456,105 USPQ 233; 235 (CCPA 1955). see MPEP § 2144.05 part II A.

4. Claim 5 stands rejected under 35 U.S.C. 103(a) as being unpatentable over US Patent 6,703,017 or by US Patent 5,425764 or US Patent 5,629,194 each in view Posselt et al (Diabetes, 1992, v.41, pages 771-775) as applied to claims 1-4 and 6-14 above, and further in view of US Patent 5,529,914 for the same reasons set forth in the previous Office Action, mailed on 01/26/05.

Applicant's arguments, filed 05/09/05 have been fully considered, but have not been found convincing .

Applicant asserts that because US Patent 6,703,017, US Patent 5,425764 US Patent 5,629,194 and Posselt et al., are not prior art and do not suggest the claimed invention they can not be used in combination with US Patent 5,529,914.

As have been discussed, supra, it is the Examiner position that the prior art of US Patent 6,703,017, US Patent 5,425764 US Patent 5,629,194 and Posselt et al., do suggest the claimed invention and thus can be used in combination with US Patent 5,529,914.

The combined references do not explicitly teachers a method of treating diabetes in a mammal comprising implanting insulin-secreting cells, wherein insulin-secreting cells are encapsulated in a biologically compatible membrane wherein said membrane comprises polyethylene glycol (PEG).

US Patent '914 teaches a new type of biocompatible membrane as a covering to encapsulate biological materials, comprising PEG that is acceptable for implants in mammalian. (see entire document, Abstract in particular). US Patent '914 teaches that various types of cells can be encapsulated in said biocompatible membrane and that said encapsulation will prevent rejection of encapsulated cells during transplantation (see column 10 in particular).

It would have been obvious to a person of ordinary skill in the art at the time the invention was made to apply the teaching of US Patent '914 to those of US Patent " 017, US Patent ' 764 , US Patent' 194 and Posselt et al., to obtain a claimed method of treating diabetes in a mammal comprising implanting insulin-secreting cells, wherein insulin-secreting cells are encapsulated in a biologically compatible membrane wherein said membrane comprises polyethylene glycol (PEG).

One of ordinary skill in the art at the time the invention was made would have been motivated to do so, because encapsulation of cells in biologically compatible membrane comprising PEG will prevent rejection of encapsulated cells during transplantation as taught by US Patent '914. Said type of biocompatible membrane can be used to substitute the different type of biocompatible membrane for successful implantation of insulin-producing cells in the method of treating diabetes taught by combined references of US Patent " 017, US Patent ' 764 , US Patent' 194 and Posselt et al. The strongest rationale for combining references is a recognition, expressly or impliedly in the prior art or drawn from a convincing line of reasoning based on established scientific principles or legal precedent, that some advantage or expected beneficial result would have been produced by their combination. In re Semaker. 217 USPQ 1, 5 - 6 (Fed. Cir. 1983). See MPEP 2144.

From the combined teaching of the references, it is apparent that one of ordinary skill in the art would have had a reasonable expectation of success in producing the claimed invention. Therefore, the invention as a whole was *prima facie* obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references, especially in the absence of evidence to the contrary.

5. No claim is allowed.

6. **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

7. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Michail Belyavskyi whose telephone number is 571/ 272-0840 The examiner can normally be reached Monday through Friday from 9:00 AM to 5:30 PM. A message may be left on the examiner's voice mail service. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Christina Chan can be reached on 571/ 272-0841 .

The fax number for the organization where this application or proceeding is assigned is 703-872-9306.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Michail Belyavskyi, Ph.D.
Patent Examiner
Technology Center 1600
June 6, 2005

CHRISTINA CHAN
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600

PTO/SB/08 Equivalent

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Multiple sheets used when necessary)

SHEET 1 OF 1

Application No.	10/823,263
Filing Date	4-13-04
First Named Inventor	Latta, Paul P.
Art Unit	1644
Examiner	Belyavskyi, Michail A.
Attorney Docket No.	LATTA.002C4

U.S. PATENT DOCUMENTS

Examiner Initials	Cite No.	Document Number *Number - Kind Code (if known)* Example: 1,234,567 B1	Publication Date MM-DD-YYYY	Name of Patentee or Applicant	Pages, Columns, Lines Where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initials	Cite No.	Foreign Patent Document *Country Code-Number-Kind Code* Example: JP 1234567 A1	Publication Date MM-DD-YYYY	Name of Patentee or Applicant	Pages, Columns, Lines Where Relevant Passages or Relevant Figures Appear	T[1]

NON PATENT LITERATURE DOCUMENTS

Examiner Initials	Cite No.	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[1]
MB	1	BINGLEY, P.J. et al. (1994) "Combined analysis of autoantibodies improves production of IDDM in islet cell antibody-positive relatives" Diabetes 43:1304(7).	
	2	BONIFACIO, E. et al. (1995) "Islet autoantibody markers in IDDM: risk assessment strategies yielding high sensitivity" Diabetologia 38:816-822.	
	3	CHRISTIE, M.R. et al. (1994) "Antibodies is islet 37k antigen, but not to glutamate decarboxylase, discriminate rapid progression to IDDM in endocrine autoimmunity" Diabetes 43:1254(6).	
	4	LEE, H.C. et al. (1995) "Relationships among 64k autoantibodies, pancreatic beta-cell function, HLA-DR antigens and HLA-DQ genes in patients with insulin-dependent diabetes mellitus in Korea" Korean J. Intern Med. (Abstract only).	
	5	TUOMILEHTO, J. et al. (1994) "Antibodies to glutamic acid decarboxylase as predictors of insulin-dependent diabetes mellitus before clinical onset of disease" Lancet 343:1383-1385.	
	6	ZIMMET, P.Z. et al. (1994) "Latent autoimmune diabetes mellitus in adults (LADA): the role of antibodies to glutamic acid decarboxylase in diagnosis and prediction of insulin dependency" Diabetic Medicine 11:299-303.	
MB	7	ZIMMET, P.Z. et al. (1994) "Autoantibodies to glutamic acid decarboxylase and insulin in islet cell antibody positive presymptomatic type 1 diabetes mellitus: frequency and segregation by age and gender" Diabetic Medicine 11:866-871.	

1688567:vr
042905

Examiner Signature	Date Considered 6/8/05

*Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] - Place a check mark in this area when an English language Translation is attached.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Paul P. Latta
Appl. No.	:	10/823,263
Filed	:	April 13, 2004
For	:	A METHOD OF TREATMENT OF DIABETES THROUGH INDUCTION OF IMMUNOLOGICAL TOLERANCE
Examiner	:	Belyavskyi, Michail A.
Group Art Unit	:	1644

AMENDMENT WITH RCE

Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In response to the Office Action mailed June 16, 2005, please amend the above-identified application as follows:

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks/Arguments begin on page 4 of this paper.

AMENDMENTS TO THE CLAIMS

1. **(Currently amended)** A method of treating diabetes in a mammal in need thereof, comprising the steps of:

implanting in said mammal a tolerizing dose of insulin-secreting cells encapsulated in a biologically compatible permselective membrane, wherein said implanting step is subcapsular, subcutaneous, intraperitoneal or intraportal; then

administering to said mammal a therapeutic dose of corresponding unencapsulated insulin-secreting cells.

2. **(Original)** The method of claim 1, wherein said mammal is a human, canine or feline.

3. **(Previously presented)** The method of claim 1, wherein said tolerizing dose is one to two orders of magnitude less than said therapeutic dose.

4. **(Original)** The method of claim 1, wherein said insulin-secreting cells are pancreatic islet cells.

5. **(Original)** The method of claim 1, wherein said membrane comprises polyethylene glycol.

6. **(Previously presented)** The method of claim 1, wherein said tolerizing and therapeutic doses comprise porcine cells.

7. **(Previously presented)** The method of claim 1, further comprising the step of administering one or more anti-inflammatory agents to said mammal prior to, at the same time as, or subsequent to administration of said therapeutic dose.

8. **(Original)** The method of claim 1, wherein said membrane has a molecular weight cutoff of about 150 kDa or less.

9. **(Original)** The method of claim 1, wherein said membrane has a pore size of less than about 0.4 μm.

10. **(Original)** The method of Claim 9, wherein said membrane has a pore size of less than about 0.2 μm.

11. **(Previously presented)** The method of Claim 1, wherein said therapeutic dose is between one and two orders of magnitude higher than said tolerizing dose.

12. **(Cancelled)**

13. **(Original)** The method of Claim 1, wherein said administering step is intraperitoneal, intraportal or subcutaneous.

14. **(Original)** The method of Claim 1, wherein said tolerizing dose is administered incrementally.

REMARKS

Claim 1 has been amended. Support for the amendment can be found in the Specification as filed, on page 4, lines 16-17 and the original Claim 12. Claim 12 has been canceled as redundant. No new matter has been introduced by this amendment. The following addresses the substance of the Office Action.

Non-obviousness

The Examiner has continued rejecting Claims 1-4 and 6-14 under 35 USC §103(a) as being allegedly obvious over USP 6,703,017 or USP 5,425,764, or USP 5,629,194 each in view of Posselt et al. (Diabetes, 1992, 41:771-775).

> Pursuant to MPEP 2143, in order to establish a *prima facie* case of obviousness three requirements must be met: First there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations.
>
> Furthermore, "Obviousness analysis requires <...> to assess invention as a whole to determine whether there was suggestion or motivation to combine prior art references, without engaging in improper "hindsight" determination <...>. <...> motivation to combine may be found in nature of problem to be solved, leading inventors to look to references relating to possible solutions to that problem" *Ruiz v. A.B. Chance Co.* 69 USPQ2d 1686-1691.

In the case of the present invention, the cited references fail to suggest all of the claim limitations, and in fact actually teach away from the presently claimed invention. As discussed in the previous response to Office Action, and as acknowledged by the Examiner, USP '017 or USP'764 or USP'194 do not teach a method for treating diabetes in a mammal comprising administration of two doses of insulin-secreting cells: one – tolerizing and one – therapeutic, wherein the tolerizing dose is at least one order of magnitude less that the therapeutic dose. The problem of rejection of the implants of the USP '017 or USP'764 or USP'194 is addressed differently: by reduction of antigenicity or by encapsulation of islet-producing stem cells in USP'017, by encapsulation of islets of Langerhans in USP'764, and by altering antigens on the cell surface of the porcine pancreatic cells or by using immunosuppressant drugs in USP'194. If a skilled artisan was still looking for other ways to solve the same problem of creating tolerance

to the implant, the publication of Posselt would point the artisan only in one direction: intrathymic implantation of a tolerizing dose of insulin-producing cells.

Posselt et al. describes implanting unencapsulated islets into various areas of the body, liver, kidney, and thymus, but the only implantation site that showed survival of the implanted cells was the thymus. As the authors stated several times in this article, thymus is considered to be an immunologically privileged site and is subject to the usual biologic characteristics of such sites, in that prior sensitization of the host with skin allografts precludes prolonged survival of intrathymic islets (page 272, right column). The experiments, performed by Posselt et al. show just that, i.e. when allogeneic islets were transplanted into the thymus of recipients that had previously rejected donor strain skin grafts, the islets were destroyed in an accelerated manner, demonstrating that the intrathymic site is readily accessible to activated T cells (page 367 left column, and page 368, right column), and that no tolerance can be achieved using this protocol. Furthermore, Posselt et al. goes on stating that the achieved tolerization to intrathymic allografts is due to their direct influence on maturing thymocytes, which are more susceptible to tolerance-inducing signals, and that such "inappropriate" presentation of antigen by nonlymphoid cells induce a state of anergy in T cells (page 373). Therefore, Posselt et al. actually teaches away from using the tolerizing dose of insulin-producing cells anywhere but thymus, and it only shows success in the absence of prior sensitization to the implant. As such, one skilled in the art would have no reasonable expectation of success in using the invention of the presently recited claims involving subcapsular, subcutaneous, intraperitoneal or intraportal implantation, and would have no motivation to do so.

The Applicant was the first one to teach that implantation of insulin-producing cells in sites other than thymus produces tolerance to the implanted cells. The Declaration of Dr. Scharp, submitted on May 6, 2005, reiterates that. The instant method as claimed in the presently amended Claim 1 specifies the non-thymus implantation sites for the tolerizing dose of encapsulated cells. This limitation is not suggested in any of the cited references.

Therefore, the cited references combined fail to provide a reasonable expectation of success or suggest all of the claim limitations. As such, the cited references fail to support a *prima facie* case of obviousness. Therefore, Claims 1-4 and 6-14 are in compliance with 35 U.S.C. 103.

The Examiner has continued rejecting Claim 5 under 35 U.S.C. 103(a) as being unpatentable over US Patent 6,703,017 or by US Patent 5,425,764, or US Patent 5,629,194 each in view Posselt et al. (1991 *Ann. Surg.* **214**:363-373) as applied to Claims 1-4 and 6-14, and further in view of USP 5,529,914. Non-obviousness of the independent Claim 1 in view of US Patent 6,703,017 or by US Patent 5,425,764, or US Patent 5,629,194 each in view Posselt et al. is asserted above. The US Patent 5,529,914 discloses a method of encapsulating cells, but it fails to cure the deficiencies of US Patent 6,703,017, US Patent 5,425,764, US Patent 5,629,194, and Posselt et al. Therefore, Claim 5 is in compliance with 35 USC §103(a).

CONCLUSION

Applicants have endeavored to address all of the Examiner's concerns as expressed in the outstanding Office Action. Accordingly, amendments to the claims, the reasons therefor, and arguments in support of the patentability of the pending claim set are presented above. In light of the above amendments and remarks, reconsideration and withdrawal of the outstanding rejections is specifically requested. If the Examiner finds any remaining impediment to the prompt allowance of these claims that could be clarified with a telephone conference, the Examiner is respectfully requested to initiate the same with the undersigned.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: August 17, 2005

By: _____

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LATTA.002C4
DEA/MXG

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/823,263	04/13/2004	Paul P. Latta	LATTA.002C4	3489

20995 7590 11/03/2005

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EXAMINER	
BELYAVSKYI, MICHAIL A	
ART UNIT	**PAPER NUMBER**
1644	

DATE MAILED: 11/03/2005

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	10/823,263	LATTA, PAUL P.	
	Examiner	Art Unit	
	Michail A. Belyavskyi	1644	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *19 August 2005*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-11,13 and 14* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-11,13 and 14* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

1. A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 08/19/05 has been entered.

Claims 1-11, 13-14 are pending.

Claims 1-11, 13-14 are under consideration in the instant application.

In view of the amendment, filed 08/19/05 the following rejections remain:

2. The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

3. Claims 1-4 and 6 –11,13 and 14 stand rejected under 35 U.S.C. 103(a) as being unpatentable over US Patent 6,703,017 or by US Patent 5,425764 or US Patent 5,629,194 each in view Posselt et al (Diabetes, 1992, v.41, pages 771-775) for the same reasons set forth in the previous Office Action, mailed on 06/16/05

Applicant's arguments, filed 08/19/05 have been fully considered, but have not been found convincing .

Applicant asserts that Posselt et al., teaches away from using tolerizing dose of insulin producing cells anywhere but thymus and it only shows success in the absence of prior sensitization to the implant.

The Examiner disagrees with Applicant's interpretation of Posselt et al. The issue raised in the previous Office Action was that it is the Examiner position that Posselet et al., teach two step stratergy: first administering a small dose of cells that induces an unresponsive state, i.e.

tolerizing dose and then administering fully therapeutic dose, at another site. Posselet et al., teach that said strategy permits the survival of pancreatic islet transplant. (see entire document, Abstract and page365 in particular). The fact that Posselet et al., implanted the first, i.e. tolerizing dose into thymus does not neglect the teaching of the advantages of using the two step process i.e. implanting first a small number of cells in one place and then implanting a therapeutic dose in different place. Posselet et al., teach that the important goal in the treatment of insulin-dependent diabetes by pancreatic islet transplantation is the development of strategies that allow permanent survival of pancreatic islet without continuous host immunosuppression. There is no indication or suggestion in Posselt et al. that only intrathymic transplantation should be performed. Posselt et al., teach that the finding that recipient bearing established intrathymic graft fail to destroy subsequent extrathymic islets either by rejection or autoimmunity argues that additional mechanism that alter systemic immune response are also involved. In other words, one skilled in the art would immediately recognized that Posselet et al., teach an advantage of two step process in the treatment of insulin-dependent diabetes by pancreatic islet transplantation. However, it is noted that the instant claims does not recited any specific place where a first tolerizing dose of insulin-secreting cells should be implanting.

US Patent '017 teaches a method of treating diabetes in a mammal comprising implanting insulin-producing cells encapsulated in a biologically compatible membrane (see entire document, Abstract and columns 6, 8, 9 –14 and Example 12 in particular) . US Patent '017 teaches that insulin producing cells are pancreatic islet cells from primary cell source (see columns 8 and 11 in particular). US Patent '017 teaches that pancreatic islet cells are from the same species as the mammal and are implanted interperitoneally into the tissue of a mammal beneath the kidney capsule (see overlapping columns 13-14 and Example 2 in particular). US Patent '017 teaches that encapsulation of said insulin-producing cells in biologically compatible membrane for success of implantation is well known in the art (see column 12 and Example 12 in particular).

US Patent '764 teaches a method of treating diabetes in a mammal comprising implanting insulin-producing cells encapsulated in a biologically compatible membrane (see entire document, Abstract and overlapping columns 5-6 in particular). US Patent '764 teaches that insulin producing cells are pancreatic islet cells (see column 1 and 4 in particular). US Patent '764 teaches that cells are implanted interperitoneally (see column 5 in particular).

US Patent '194 teaches a method of treating diabetes in mammal comprising implanting insulin-producing cells encapsulated in a biologically compatible membrane (see entire document, Abstract overlapping columns 7-8 , 12 and Example II in particular). US Patent '764 teaches that insulin producing cells are pancreatic islet cells (see column 8 in particular). US Patent '764 teaches that cells are implanted intaportal (see column 7 in particular). US Patent '194 teaches administration of one or more anti-inflammatory agent at the dosage sufficient to achieve the desired therapeutic effect. US Patent '194 teaches that said agent can be administered prior to

at the same time or subsequent to administration of insulin-producing cells (see overlapping columns 14-15 in particular).

US Patent '017 or US Patent ' 764 or US Patent '194 does not explicitly teaches a method of treating diabetes in a mammal comprising administration two doses of insulin-secreting cells one tolerizing and one curative wherein tolerizing doze is one order less than curative.

As has been discuused supra, it is the Examiner position that Posselet et al., teach that the important goal in the treatment of insulin-dependent diabetes by pancreatic islet transplantation is the development of strategies that allow permanent survival of pancreatic islet without continuous host immunosuppression. Posselet et al., further teach a strategy comprising two step process : first administering a small dose of cells that induces an unresponsive state, i.e. tolerizing dose and then administering fully therapeutic dose, at another site (see entire document, Abstract in particular). Posselet et al., teach that said strategy permits the survival of pancreatic islet transplant.

It would have been obvious to a person of ordinary skill in the art at the time the invention was made to apply the teaching of Posselt at al. to those of US Patent '017 or US Patent ' 764 or US Patent '194 to obtain a claimed method of treating diabetes in a mammal comprising administration two doses of insulin-secreting cells one tolerizing and one curative wherein tolerizing doze is one order less than curative

One of ordinary skill in the art at the time the invention was made would have been motivated to do so, because a strategy comprising two step process : first administering a small dose of cells that induces an unresponsive state, i.e. tolerizing dose and then administering fully therapeutic dose, at another site permits the survival of pancreatic islet transplant as taught by Posselet et al. Said strategy can used in the method of treating diabetes in a mammal, comprising implanting pancreatic islet, taught by US Patent '017 or US Patent ' 764 or US Patent '194. The strongest rationale for combining references is a recognition, expressly or impliedly in the prior art or drawn from a convincing line of reasoning based on established scientific principles or legal precedent, that some advantage or expected beneficial result would have been produced by their combination. In re Semaker. 217 USPQ 1, 5 - 6 (Fed. Cir. 1983). See MPEP 2144.

From the combined teaching of the references, it is apparent that one of ordinary skill in the art would have had a reasonable expectation of success in producing the claimed invention. Therefore, the invention as a whole was *prima facie* obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references, especially in the absence of evidence to the contrary.

Claims 8-11, 13 and 14 are included because it would be conventional and within the skill of the art to : (i) determine the proper pore size for the permselective membrane or (ii) to determine the optimum dosage and means of administration of insulin-secreting cells in an absent of a showing of unobvious property. Moreover, Applicant acknowledge that one of ordinary skill in the art can readily determine the proper pore size for the permselective membrane (see page 8, line 13-20 of the instant Specification in particular). Further, it has been held that where the general conditions of a claim are disclosed in the prior art, discovering the optimum or workable ranges or means of administration involves only routine skill in the art. *In re Aller*, 220 F2d 454,456,105 USPQ 233; 235 (CCPA 1955). see MPEP § 2144.05 part II A.

4. Claim 5 stands rejected under 35 U.S.C. 103(a) as being unpatentable over US Patent 6,703,017 or by US Patent 5,425764 or US Patent 5,629,194 each in view Posselt et al (Diabetes, 1992, v.41, pages 771-775) as applied to claims 1-4 and 6-14 above, and further in view of US Patent 5,529,914 for the same reasons set forth in the previous Office Action, mailed on 06/16/05

Applicant's arguments, filed 08/19/05 have been fully considered, but have not been found convincing .

Applicant asserts that because US Patent 6,703,017, US Patent 5,425764 US Patent 5,629,194 and Posselt et al., are not prior art and do not suggest the claimed invention they can not be used in combination with US Patent 5,529,914.

As have been discussed, supra, it is the Examiner position that the prior art of US Patent 6,703,017, US Patent 5,425764 US Patent 5,629,194 and Posselt et al., do suggest the claimed invention and thus can be used in combination with US Patent 5,529,914.

The combined references do not explicitly teachers a method of treating diabetes in a mammal comprising implanting insulin-secreting cells, wherein insulin-secreting cells are encapsulated in a biologically compatible membrane wherein said membrane comprises polyethylene glycol (PEG).

US Patent '914 teaches a new type of biocompatible membrane as a covering to encapsulate biological materials, comprising PEG that is acceptable for implants in mammalian. (see entire document, Abstract in particular). US Patent '914 teaches that various types of cells can be encapsulated in said biocompatible membrane and that said encapsulation will prevent rejection of encapsulated cells during transplantation (see column 10 in particular).

It would have been obvious to a person of ordinary skill in the art at the time the invention was made to apply the teaching of US Patent '914 to those of US Patent " 017, US Patent ' 764 , US Patent' 194 and Posselt et al., to obtain a claimed method of treating diabetes in a mammal comprising implanting insulin-secreting cells, wherein insulin-secreting cells are encapsulated in a biologically compatible membrane wherein said membrane comprises polyethylene glycol (PEG).

One of ordinary skill in the art at the time the invention was made would have been motivated to do so, because encapsulation of cells in biologically compatible membrane comprising PEG will prevent rejection of encapsulated cells during transplantation as taught by US Patent '914. Said type of biocompatible membrane can be used to substitute the different type of biocompatible membrane for successful implantation of insulin-producing cells in the method of treating diabetes taught by combined references of US Patent " 017, US Patent ' 764 , US Patent' 194 and Posselt et al. The strongest rationale for combining references is a recognition, expressly or impliedly in the prior art or drawn from a convincing line of reasoning based on established scientific principles or legal precedent, that some advantage or expected beneficial result would have been produced by their combination. In re Semaker. 217 USPQ 1, 5 - 6 (Fed. Cir. 1983). See MPEP 2144.

From the combined teaching of the references, it is apparent that one of ordinary skill in the art would have had a reasonable expectation of success in producing the claimed invention. Therefore, the invention as a whole was *prima facie* obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references, especially in the absence of evidence to the contrary.

5. No claim is allowed.

6. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Michail Belyavskyi whose telephone number is 571/272-0840. The examiner can normally be reached Monday through Friday from 9:00 AM to 5:30 PM. A message may be left on the examiner's voice mail service. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Christina Chan can be reached on 571/272-0841.

The fax number for the organization where this application or proceeding is assigned is 571/273-8300

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Michail Belyavskyi, Ph.D.
Patent Examiner
Technology Center 1600
October 28, 2005

PTO/SB/08 Equivalent

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Multiple sheets used when necessary)

SHEET 1 OF 1

Application No.	10/823,263
Filing Date	April 13, 2004
First Named Inventor	Latta, Paul P.
Art Unit	1644
Examiner	Michail A. Belyavskyi
Attorney Docket No.	LATTA.002C4

U.S. PATENT DOCUMENTS

Examiner Initials	Cite No.	Document Number *Number - Kind Code (if known)* Example: 1,234,567 B1	Publication Date MM-DD-YYYY	Name of Patentee or Applicant	Pages, Columns, Lines Where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initials	Cite No.	Foreign Patent Document *Country Code-Number-Kind Code* Example: JP 1234567 A1	Publication Date MM-DD-YYYY	Name of Patentee or Applicant	Pages, Columns, Lines Where Relevant Passages or Relevant Figures Appear	T[1]

NON PATENT LITERATURE DOCUMENTS

Examiner Initials	Cite No.	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[1]
MB	1	ATKINSON M.A. et al. (1999) "The NOD mouse model of type 1 diabetes: as good as it gets?" Nature Medicine 5:601-604.	

1776045:vr061705

Examiner Signature	Date Considered 10/21/05

*Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

T[1] - Place a check mark in this area when an English language Translation is attached.

INDUCTION OF IMMUNOLOGICAL TOLERANCE

Related Applications

This application is a continuation of Application Serial No. 10/660,924 filed September 12, 2003 which is a continuation of Application Serial No. 09/226,742 filed January 7, 1999 now abandoned which was a continuation of Application Serial. No. 09/049,757 filed March 27, 1998 now abandoned, which was a continuation of Application Serial No. 08/736,413 filed on October 24, 1996 now abandoned, which claims the benefit of priority under 35 U.S.C. 119(e) of Provisional Application No. 60/005,877 filed October 26, 1995.

Field of the Invention

The present invention relates to the induction of immunological tolerance to foreign cells, tissues and organs. More specifically, the invention relates to implantation of a tolerizing dose of cells or tissues encapsulated in a membrane in a mammal to establish immunological tolerance thereto.

Background of the Invention

For some human diseases, including heart and liver failure, organ transplantation is the only alternative to certain death. While there were only 4,843 organ donors in the U.S. in 1993, there were 2,866 heart and 3,040 liver failure patients on the waiting list for these organs (*UNOS Update*, 10(2), 1994). Thus, because of timing and tissue matching problems, many patients die each year for lack of an available organ. For those lucky enough to receive an organ, the results are still less than ideal. The transplant procedure constitutes major surgery which is associated with attendant risks and is exceedingly expensive. After the surgery, the patient must be placed on a regimen of immunosuppressive drugs to keep the immune system from destroying the transplanted organ. As a consequence, the patient's entire immune system is suppressed for the rest of his life, significantly lowering his defenses against other serious disease threats such as infections, viruses or cancers.

For other diseases including kidney failure, pancreas failure and cystic fibrosis, transplantation has a lower mortality and morbidity rate than any alternative therapy. Even with its attendant problems of organ scarcity, surgical risk, high cost and permanent immunosuppression, for some of these cases it is still a more practical therapy than any alternative. The physician's choice in these cases is dependent on many variables including age, general health, severity of the condition, availability of organs and others factors. In 1994, there were 25,033 patients on the waiting list for human kidneys, 181 for pancreases and 1,250 for lungs (*UNOS Update*, 10:2, 1994).

For still other diseases, transplantation is known to be effective, although its attendant problems preclude its practical therapeutic use. This is true for many of the kidney, pancreas and lung patients described above. It is also true where whole pancreas transplantation can cure diabetes or liver transplantation can cure hemophilia but the risks outweigh the rewards.

Recently, for certain disease states; tissue transplants, as opposed to whole organ transplants, have been shown to be therapeutic in animals and even in man (Scharp et al., *Transplantation*, 51:76-85, 1991). Tissue transplantation requires full immunosuppression and carries the same risks and problems as already discussed for whole organ immunosuppression. The following treatments address the rejection of the transplanted tissue.

One implantation method involves pre-inoculation in the thymus with a small dose of cells, full temporary immunosuppression, then a full therapeutic dose at another site (Posselt et al., *Annals of Surgery*, 214:363-373, 1991). First, this has only been shown to work in rodents to date. No large animal or human test has been successful. Second, the human adult thymus is shrunken and may not be practical to treat with an adequate pre-dose. Third, the immunosuppression step, while temporary, does subject the patient to risks for that period of time. Fourth, it is not known whether a fully therapeutic dose will be tolerated, (i.e. not rejected) in humans. Fifth, this procedure may not protect against autoimmune destruction even if it does prevent rejection.

Another method of preventing rejection is irradiation of the recipient's bone marrow immune cells, implantation of bone marrow cells from the donor, then implantation of a full therapeutic dose of tissue or organ from the same donor (Illstad et al., *J. Exp. Med.*, 174:467-478, 1991). First, this has not been shown to work for tissue transplants in humans. Second, irradiation of immune cells, either partial or whole body, carries serious risks. Third, it is not known if the immune system will adequately protect from other threats. Fourth, it is not known if the method will protect from both rejection and autoimmune destruction in those disease states.

A further method of treatment to prevent rejection is by using monoclonal antibodies to suppress certain parts of the immune system (Andersson et al., *J.*

Autoimmun., 4:733-742, 1991). These tests have only been performed in rodents so it is not known if they would succeed in humans. Also, it is not known if the proper monoclonal antibody could be identified and created for each different disease state. In addition, the overall affect of these agents on the human immune system is not known.

Still another method of preventing rejection is encapsulation of the transplanted tissue in a semi-permeable membrane device which allows oxygen, nutrients and other small molecules to pass but prevents entry of large immune system cells (Lacy et al., *Science*, 254:1782-1784, 1991; Sullivan et al., *Science*, 252:718-721, 1991). There are several unresolved problems associated with this method. First, none of these devices has been shown to protect a therapeutic transplant in humans. To be suitable for human use, the material must be biocompatible; it must be sufficiently strong to last a long time when implanted; its porosity must be exactly correct to allow survival and function of the enclosed cells while keeping out cells and perhaps antibodies of the immune system; and finally, the device itself must be large enough to contain enough cells for a fully therapeutic implant and yet small enough to allow for some reasonable method of implantation which causes no damage to other internal organs.

To date, there has been very little effort to use transplantation as a potential prevention of disease due to all of the problems associated with transplantation as previously described. In addition, it is not yet known where transplantation can actually prevent a disease from occurring other than the obvious case of whole organ failures. Moreover, for many disease states, it is not known who will be afflicted. There is some evidence that interventional transplantation can have some preventive effect in rodents (Miller et al., *J. Neurol. Immunol.*, 46:73-82, 1993; van Vollenhoven et al., *Cell. Immunol.*, 115:146-155, 1988). Thus, a major role for preventive transplantation has not been investigated.

Summary of the Invention

One embodiment of the invention is a method of creating immunological tolerance to foreign cells, tissues or organs in a mammal, comprising the step of implanting in the mammal a tolerizing dose of foreign cells or tissue encapsulated in a biologically compatible permselective membrane. The method may additionally

comprise the step of administering to the mammal a curative dose of corresponding unencapsulated cells, tissue or organ. Advantageously, the mammal is a human, canine or feline. Preferably, the tolerizing cells are insulin-secreting cells; more preferably, they are pancreatic islet cells. According to one aspect of this embodiment, the membrane comprises polyethylene glycol. Preferably, the curative dose is between one and two orders of magnitude greater than the tolerizing dose. Advantageously, the tolerizing and curative doses are from the same species as the mammal. Alternatively, the tolerizing and curative doses are from a species different from the mammal. Preferably, the tolerizing and curative doses are porcine. The method may further comprise the step of administering one or more anti-inflammatory agents to the mammal prior to, at the same time as, or subsequent to administration of the curative dose. Preferably, the membrane has a molecular weight cutoff of about 150 kDa or less. Alternatively, the membrane has a pore size of about 0.4 μm or less. The membrane may also have a pore size of about 0.2 μm or less. Advantageously, when the tolerizing and curative doses are from a different species, the membrane has a molecular weight cutoff of about 150 kDa or less. Preferably, the tolerizing step is subcapsular, subcutaneous, intraperitoneal or intraportal and the curative step is intraperitoneal, intraportal or subcutaneous. The tolerizing dose may also be administered incrementally.

The present invention also provides a method of treating diabetes in a mammal in need thereof, comprising the steps of:

implanting in the mammal a tolerizing dose of foreign insulin-secreting cells encapsulated in a biologically compatible permselective membrane; then

administering to the mammal a curative dose of corresponding unencapsulated insulin-secreting cells.

Preferably, the mammal is a human, canine or feline. Advantageously, the tolerizing dose is one to two orders of magnitude less than the curative dose. In another aspect of this preferred embodiment, the membrane comprises polyethylene glycol. Advantageously, the insulin-secreting cells are pancreatic islet cells. Preferably, the mammal and the insulin-secreting cells are from the same species. Alternatively, the mammal and the insulin-secreting cells are from different species. Preferably, the

tolerizing and curative doses are porcine. The method may further comprise the step of administering one or more anti-inflammatory agents to the mammal prior to, at the same time as, or subsequent to administration of the curative dose. Advantageously, the membrane has a molecular weight cutoff of about 150 kDa or less. Alternatively, the membrane has a pore size of less than about 0.4 μm.

Brief Description of the Drawings

Figure 1 is plane view illustrating the key properties of the membrane enclosing the cells. The membrane may be configured into many different device designs.

Figure 2 is a plane view of one design of the invention, wherein two layers of the membrane are used in a flat sheet configuration where cells are "sandwiched" in between the two membranes and then the ends are sealed.

Figure 3 is a tubular view of one design of the invention, wherein the membrane is cast or rolled into a tubular configuration. The cells are loaded in the lumen and the ends are sealed.

Figure 4 is a spherical view of one design of the invention, wherein the membrane is cast in a spherical configuration and cells may be encased one in each device (microcapsule) or many in a device (macrocapsule).

Figure 5 is a graph showing blood glucose levels in mice implanted with a tolerizing dose of 100 encapsulated NIT insulinoma aggregates.

Figure 6 is a graph showing blood glucose levels in mice implanted with a tolerizing dose of 50 encapsulated NIT insulinoma aggregates.

Figure 7 is a graph showing blood glucose levels in non-tolerized control mice.

Detailed Description of the Preferred Embodiments

Goals of the Invention

The problems discussed in the foregoing Background of the Invention have previously not been solved for either micro or macroencapsulation of cells in humans. The present invention overcomes these problems associated with transplantation. Thus, one goal of the invention is to eliminate the critical problems of transplantation in cases where whole organ transplantation is the only alternative to certain death. These are cases of heart or liver failure. The major advantage of the invention process

for this application is that it eliminates the shortage of organs for the patients by making animal organs acceptable in humans. While there are only about 4,800 human organ donors in the U.S. each year, the supply of animal organs for transplant is not limited. The reason that animal organs are not presently used is that they are acutely rejected when transplanted into humans even with immunosuppression. Second, continuous immunosuppression is not required in the process of the invention, thus eliminating the risk of exposing the patient to other serious diseases while the immune system is suppressed. Third, the cost of organ transplantation is drastically reduced because of the unlimited supply of organs and because the continuous use of immunosuppressive drugs is not required.

A second goal of the invention is to make organ transplantation a safe, effective, practical therapy for those cases of disease where it is known now to be therapeutic but the risks associated with it prevent its widespread therapeutic use. Examples of these disease cases are kidney failure, pancreas failure and cystic fibrosis (lung failure). In these cases the advantages of the process of the invention eliminate the major obstacles. First, by making animal organs tolerated in humans the shortage of organs for these transplant needs is solved. Second, by eliminating the need for continuous immunosuppression, these patients are not exposed to other serious disease threats without a fully functioning immune system. Third, because of plentiful organs and no continuous immunosuppression, the cost of this transplant procedure would be greatly reduced.

A third goal of the invention is to make cell or tissue transplants, as opposed to whole organ transplants, a practical therapy in cases where cells or tissue alone can cure a disease state by providing a lacking or deficient protein, enzyme or peptide. Examples of these cases are insulin-secreting islet cells for Type I diabetes, Factor VIII-secreting hepatic cells for hemophilia, dopamine-secreting adrenal chromaffin cells for Parkinson's disease and collagen for arthritis. A significant advantage of the process of the invention for these cases is that animal tissue or genetically engineered tissue expressing an absent or deficient protein of interest can be used if human tissue is scarce. In addition, cell types other than the normal protein-secreting cells can be engineered to secrete the protein of interest. For example, myoblasts can be

engineered by standard methods to secrete insulin. The use of such cells is also within the scope of the present invention. Continuous immunosuppression is not needed to protect the transplanted tissue and the costs would be reduced. Thus, even if pre-inoculation into the thymus with immunosuppression or irradiation of bone marrow with immunosuppression or monoclonal antibodies could be identified and produced for many disease states or encapsulation of fully therapeutic doses of tissue in some membrane device can overcome many technical problems, the process of the invention is a safer and more practical therapy than any of these.

A fourth goal of the invention is the treatment of autoimmune diseases including diabetes, Alzheimer's, arthritis, multiple sclerosis, myasthenia gravis and systemic lupus erythematosus. In these diseases, the body's immune system attacks and destroys one's own tissue. By using the process of the invention, the immune system can be induced to accept grafted tissue or organs to replace those that have been destroyed without the autoimmune destruction of the newly transplanted graft. The advantage of this process is that organ rejection and autoimmune destruction are two completely different phenomena so that even with systems that may prevent rejection, in autoimmune diseases the grafts may still be destroyed by a different means. The process of the invention addresses both problems.

A fifth goal of the invention is to make transplantation a practical therapy to prevent certain diseases from ever occurring, as well as treating existing diseases as previously discussed. The advantage of the process that makes this possible is the immunomodulation effect which stops or prevents the immune system from destruction of self tissue. Thus, for all autoimmune disorders, the process can be used to intervene in the course of the disease at a critical point before the immune system is initiated into self-destruction of tissue that is necessary for normal body function.

As will be apparent from the ensuing detailed description of the invention, the present invention meets all of these goals. Additionally, the present invention also provides a number of advantages which would not have been readily apparent to one having ordinary skill in the art.

Overview

The present invention is a two step process. In the first step, a small number of cells or tissue is implanted into a mammal inside a device made of a biocompatible "permselective" membrane which protects the implanted cells from the mammal's immune system while at the same time allowing the cells to survive. A permselective membrane is one having a pore size selected so that it is small enough to prevent the entry of immunological factors such as cells or antibodies, yet large enough to allow the free passage of oxygen, nutrients and other molecules needed to sustain the transplanted cells. In addition, the membrane pores must allow the passage of antigens which are shed from the transplanted cells and prevent the entry of large immune system cells and antibodies. In a preferred embodiment, the mammal is a human. Alternatively, the mammal is a canine or feline.

One of ordinary skill in the art can readily determine the proper pore size for the permselective membrane for any particular application of the present invention. It is preferable to use the largest pore size possible to prevent the entry of the undesirable elements because the larger pores allow better diffusion of the desirable elements such as nutrients and oxygen across the membrane. Smaller pore sizes (e.g. those excluding molecules greater than 100,000 daltons) are not necessarily a problem for diffusion as has been shown in long-term survival of cells in a 50,000 dalton membrane *in vivo* implant (Lacy et al., *Science* 254:1782-1784, 1991).

Antigens shed from the transplanted cells pass through the permselective membrane into the body of the recipient where they are fully exposed to the immune system. The immune system will recognize these antigens as "foreign" and destroy them. This process will continue for some time with the immune system constantly destroying the shed antigens but not able to destroy the source which is the cells protected in the encapsulation device. In time, the immune system will begin to become tolerant of these antigens because they do no actual damage in the body and the constant source cannot be destroyed. At this time, the immune system is tolerant to that particular cell type from that particular donor.

Next, the second stage of the process is enacted. Now a fully therapeutic (curative) dose of cells, tissue or whole organ from the same donor as the tolerizing

dose is implanted in the recipient for cure of the disease. Since this implant, whether cells, tissue or organ, is from the same donor as the small dose, it is recognized by the immune system as "self" and a rejection response is not elicited. The immune system is fully tolerant to the new implant. In one embodiment, the tolerizing dose is given as a single (bolus) dose. Alternatively, the tolerizing dose may be administered incrementally over several weeks or months. In a preferred embodiment, the incremental tolerizing dose is the same as the bolus dose, only spread out in even increments. In another embodiment, the total incremental tolerizing dose is one to three times the bolus tolerizing dose. As for the bolus tolerizing dose, the incremental tolerizing dose is typically one to two orders of magnitude lower than the curative dose.

In addition to eliminating continuous immunosuppression, this process makes animal organs and cells available for human implants (xenografts). Presently, these organs or tissues are acutely rejected in humans because of the wide immunological barriers between the species. With the process of the invention, even animal tissue will be tolerated because tolerance is induced gradually over time. The availability of animal organs for human use will save many thousands of lives each year which are now lost due to the shortage of available human organs for transplantation. In addition, this process will allow transplant therapy for autoimmune diseases such as diabetes, arthritis, myasthenia gravis and multiple sclerosis. This is possible because as the immune system is tolerized to the new tissue by the initial small implant, the self-destructive autoimmune process is suppressed. So, for diseases requiring organs or cellular transplants, this process eliminates current shortages by making unlimited supplies of animal organs and cells available, eliminates the need for continuous immunosuppression, and protects the transplants from both rejection and autoimmune destruction. One particularly preferred source of xenograft cells or tissue for both the tolerization and curative steps is porcine cells or tissue.

Even with the tolerizing effect of the xenograft, because of the wide species differences, an initial inflammatory reaction may occur in response to the curative dose. Thus, in one embodiment of the invention, the xenograft recipient is administered one or more anti-inflammatory agents. The anti-inflammatory agent is

administered either systemically or locally at the implantation site. The agent may be administered prior to the implant, at the time of implantation or subsequent to the implant for a time necessary to overcome the initial inflammatory reaction. The agents may be over-the-counter preparations such as acetaminophen or ibuprofen, or a specific immunosuppressive agent such as Cyclosporine (Sandoz) or Imuran (azathioprine, Burroughs-Wellcome). The agent may also block the binding of a particular antigen such as CTLA4Ig (Bristol Myers Squibb). The amount of anti-inflammatory agent to be administered is typically between about 1 mg/kg and about 10 mg/kg. The extent of inflammation will determine whether the administration of such an agent(s) is necessary. The need for such agents is only temporary and not required for the ongoing survival and function of the transplant.

The process of the invention can also be used to prevent certain diseases, particularly autoimmune disorders. In these cases the process is as follows. First, patients at high risk for the disease or already in the very early phase of the disease are identified. At the critical time of the onset, the process is intervened with the small encapsulated tissue. For example, islets are used for Type I diabetes and collagen is used for arthritis. This implant of foreign tissue immediately diverts the attention of the immune system to the new foreign invader and it begins the process to destroy this new threat. Because of this diversion, the process of self-destruction of desirable tissue that was just beginning is suppressed, then abandoned, then forgotten. It is, in essence, "switched off" and the damage is prevented.

Implantation of cells to treat existing diseases

The first step of this method involves acquiring small amounts of cellular tissue for the initial tolerizing implant. The method in which tissue is obtained depends on the type of tissue needed, the source of the tissue, the donor, and the amount of tissue needed. These methods are generally well known by those skilled in the art of tissue digestion, separation, purification, culture, and the like. The following examples are only used to illustrate that these methods are readily available.

Islet cells for treatment of diabetes

Islets are small clusters of cells located in the pancreas of mammals. They are composed of alpha cells which make and secrete somatostatin, beta cells which make

insulin, delta cells which make glucagon and other cells which make other proteins. To isolate the islet cells which make up only 1-2% of the pancreas from the surrounding acinar tissue, the digestive enzyme collagenase is used. This process is described by Ricordi (*Diabetes* 37:413-410, 1988, hereby incorporated by reference). Once the islets are obtained, they are purified from acinar cells and can then be implanted fresh, cultured for extended periods, cryopreserved indefinitely or encapsulated.

For use in human treatments, primary islet cells are obtained from human cadaver donors or from suitable mammalian sources such as rat, cow, or pig. For use of animal tissue in humans, it is desirable to assure safety of the animal source by using specific pathogen-free (SPF) or gnotobiotic colonies or herds of animals. As an alternative to a primary cell source, an engineered cell line which is genetically altered to produce the proper regulated amounts of insulin, glucagon, somatostatin, etc. is also suitable for treatment of diabetes.

Adrenal chromaffin cells for Parkinson's disease, Alzheimer's and Huntington's disease

Adrenal chromaffin cells have multiple applications. They secrete the neurotransmitter dopamine for amelioration of Parkinson's disease, fibroblast growth factor, and can be engineered to secrete nerve growth factor which will counter degeneration and cell death in Alzheimer's and Huntington's disease. A collagenase digestion method of isolating adrenal chromaffin cells from the adrenal gland is described by Livett (*Physiol. Rev.* 64:1103-1161, 1984). Human or other mammalian sources can be appropriate sources of this tissue.

Moreover, mammalian cells can also be genetically engineered to secrete certain proteins or peptides whose absence or deficiency is the cause of various genetic diseases (i.e. adenosine deaminase deficiency). In addition, such cells can also be engineered to secrete various cytokines and growth factors for the treatment of viral infections (i.e., interferon-γ) and cancer (i.e., interleukin-2). Hormone deficiencies can also be treated by this method. Mammalian cells are transfected with an expression vector containing a gene encoding such a therapeutic protein or peptide. These expression vectors are constructed using standard methods well known to one of

ordinary skill in the art. A tolerizing dose of these cells is encapsulated as described herein and implanted into a mammal. Two to three weeks later, a curative dose of the same cells is implanted into the mammal. The cells are no longer recognized as foreign, are not destroyed by the host immune system and continue to secrete the desired therapeutic protein.

Other conditions treatable by encapsulated cells producing peptides, proteins or other therapeutic agents include hypoparathyroidism (thyroid hormone), hyperadrenocorticalism (adrenocorticotrophic factor), dwarfism (growth hormone), Gaucher's disease (glucocerebrosidase), Tay-Sachs (hexosaminidase A) and cystic fibrosis (cystic fibrosis transmembrane regulator). In addition, cells expressing stimulatory or inhibitory cytokines can be encapsulated, resulting in stimulation or inhibition, respectively, of a particular cell type. For example, erythropoietin stimulates red blood cell production, interleukin-2 stimulates the proliferation of tumor-infiltrating lymphocytes and interferons inhibit certain types of tumor cells. Other conditions contemplated for treatment using the method of the present invention include amyotrophic lateral sclerosis, Alzheimer's disease, Huntington's Chorea, epilepsy, hepatitis, anxiety, stress, pain, addiction, obesity, menopause, endometriosis, osteoporosis, hypercholesterolemia, hypertension and allergies.

Other cell sources and methods for other diseases

Other cell/tissue sources and methods include collagen recovery from chicken for prevention and treatment of arthritis, Schwann cells from myelin tissue for prevention and treatment of neural degeneration and Factor VIII from liver hepatocytes for treatment and prevention of hemophilia.

The amount of cells or tissue necessary for the initial tolerizing implant will vary depending on the disease, site, source, whether the tissue is primary or immortalized and other factors. Generally, the tolerizing dose is one or two orders of magnitude less than a full dose implant. For example, in diabetes it usually takes between about 10,000 - 20,000 islets/kg of body weight to provide adequate insulin production for normoglycemia. Accordingly, the initial implant dose for tolerization is about 100 - 2,000 islets/kilogram of body weight. Although the size of these doses are not known for all disease states, they can be optimized using routine dose/response

experiments well known to one of ordinary skill in the art. In general, between about 100 cells/kg body weight and about 5,000 cells/kg body weight are suitable for tolerization. The corresponding curative doses are between about one and two orders of magnitude higher than these numbers.

Preparation of encapsulation device, loading of cells and implantation

The membrane for the device is chosen for the application needed based on its biocompatability, permeability, strength, durability, ability to be manipulated and other important considerations. A number of materials have already been shown to be acceptable for implants in mammals. Examples of some of these materials are PAN/PVC acrylic co-polymers, hydrogels such as alginate or agarose, mixed esters cellulose, polytetrafluoroethylene (PTFE)/polypropylene (Lum et al., *Diabetes* 40:1511-1516, 1991; Aebischer et al., *Exp. Neurol.*, 111:269-275, 1991; Liu et al., *Hum. Gene Ther.* 4:291-301, 1993; Hill et al., *Cell Transplantation* 1:168, 1992, all hereby incorporated by reference) and polyethylene glycol (PEG) conformal coating configurations (U.S. Patent No. 5,529,914, hereby incorporated by reference).

A critical factor is the pore size that can be produced in the material chosen. For example, PEG macromers can vary in molecular weight from 0.2 - 100 kDa. The degree of polymerization, and the size of the starting macromers, directly affect the porosity of the resulting membrane. Thus, the size of the macromers are selected according to the permeability needs of the membrane. It is believed that for xenograft transplants (animal to human), antibodies of the immune system and complement are involved in rejection (Bachet al. *Transplantation* Overview 6(6):937-947, 1991). In this case, a pore size (molecular weight cutoff) of 150 kDa or smaller is desired to prevent the passage of the smallest immune antibody (IgG) through the pores of the membrane capsule. Thus, the application and its conditions will determine the choice of membrane material from many available alternatives. Likewise, the configurations of the device will be determined by the application. For purposes of encapsulating cells and tissue in a manner which prevents the passage of antibodies across the membrane but allows passage of nutrients essential for cellular metabolism, the preferred starting macromer size is in the range of about 3 kDa to 10

kDa, with the most preferred being about 4 kDa. Smaller macromolecules result in polymer membranes of a higher density with smaller pores.

It is also believed that for allografts (human to human), only entry of immune system cells must be blocked to prevent rejection of transplanted tissue or organs (Auchincloss, Jr., *Transplantation* Overview 46(1):1-20, 1988. In addition, it is also desirable to exclude other cells, the smallest of which are red blood cells which have a size of about 7 μm. Accordingly, a membrane having a molecular weight cutoff of about 150 kDa is also suitable for encapsulation of allograft cells or tissue because such membranes will prevent entry of such cells. In an alternative embodiment, the pore size for allografts is about 0.4 μm or less to prevent the entry of immune and non-immune cells into the device. Cells can also extend processes ("arms") which can enter openings having a size of about 0.2 μm. Thus, in another preferred embodiment, the pore size is about 0.2 μm or less. In a most preferred embodiment, the pore size is as small as possible to exclude entry of detrimental components, but allows cell survival by permitting vital molecules such as nutrients, proteins and oxygen to freely pass through the permselective membrane. A desired pore size may be obtained by adjusting the crosslink density and length of PEG segments by one of ordinary skill in the art without undue experimentation.

If retrieval of the initial implant is unnecessary or undesirable, then a suitable configuration may be microcapsules where only a few or even single cells are each enclosed in separate membranes. Because of the small volume in this case, the microcapsules may simply be injected into one of many sites for the implant. If it is desirable to retrieve or reload the device or larger numbers of cells are necessary, a "macrocapsule" may be constructed wherein many cells are enclosed together inside one membrane. In this case, it has been shown that the environment inside the macrocapsule may need special conditions to allow the cells to survive. For example, an alginate matrix has been used to immobilize islet cells and prevent their aggregation and subsequent central necrosis (Lacy et al., *Science*, 254:1782-1784, 1991).

For other cell types a different environment may be needed. The macrocapsule may be of any shape that is practical. Examples of shapes commonly used by those

skilled in the art are: 1) flat sheet "sandwiches" where two layers of the membrane are top and bottom on the cells and the ends are sealed by heat welding, gluing, or other known means (Fig. 2). This method provides a large surface area for membrane exposure to the host systems and generally short diffusion distances which helps transport substances across the membrane; 2) A tubular membrane formed by co-extrusion or rolling a flat sheet into a tube and sealing the ends (Fig. 3).

The cells can be placed inside the lumen at the same time the membrane is formed if co-extrusion is employed. If the tube is made first, the cells are loaded by syringe or other means and the ends are sealed by heat welding, gluing or other known means. As previously discussed, various matrices may be employed as needed by the enclosed cells. The tubes can be any suitable length and may be joined at the ends (potted) or woven if multiple tubes are used; 3) a spherical shape (Fig. 4) which has a large surface area compared to its volume and is efficient in some applications.

These are only illustrative examples of how membranes may be configured into devices to hold cells. One of ordinary skill in the art will appreciate that many more configurations are possible, thus providing great flexibility for many conceivable applications.

The loaded devices are then implanted into patients in need of therapy. The method of implantation, site and duration are dependent on the disease being treated. For example, in diabetes it is desirable to have the shed antigens processed by the liver. Therefore, implantation in the peritoneum where the portal circulation would carry the antigens directly to the liver (intraportal) is a preferred site. Alternatively, if the dose is a small enough volume (i.e. 10 µl or less), direct injection into the portal vein is preferred. Other implantation sites include under the kidney capsule and subcutaneous implantation.

For Parkinson's disease, the cells should be processed first in the brain. Thus implanting into the interstitial region of the brain is a preferred site. For each site, the method of implantation may be different. For example, intraperitoneal placement of a device for diabetes may be performed by a minimally invasive laparoscopic procedure. To place a device in the brain, the neurosurgeon commonly uses stereotaxic instruments to ensure exact placement. For a subcutaneous implant, a

small incision to allow a trocar to be inserted may be used. For each preferred site, those skilled in the art will recognize the most efficient method of implantation.

Once implanted, the cells are left in place for a period of time during which tolerization will occur. This time period will vary depending on the disease treated, whether an allograft or a xenograft transplant is being used, site of the implant, and other factors. Generally, tolerization requires from a few weeks to a few months. During this time, the transplanted cells constantly shed antigens from their surface. These antigens comprise a variety of small molecules which are constantly being replaced by living cells The antigens can pass freely out of the pores of the membrane and into the recipient at the locations of the implant and eventually into the circulatory system. The immune system immediately recognizes these antigens as "foreign" and initiate its mechanisms to protect the recipient from the intruder. These mechanisms are complex and not completely understood. Generally, it is believed that if the foreign matter is from a closely related species (allogeneic), cells of the immune system play the primary role in the immunological response. These cells include T-cells, macrophages, neutrophils, and natural killer cells which seek out the source of the invasion and destroy the foreign matter. If the foreign matter is a transplanted xenogeneic organ, preformed antibodies cause hyperacute (within minutes) reaction and rejection of the organ. If the foreign matter is xenogeneic cells or tissue, the antigen may not be presented and the preformed antibodies may not be the primary mechanism of rejection. Instead, macrophages stimulate killer T-lymphocytes and later (8-10 days) antibody stimulation causes final rejection of cells or tissue.

In the present invention, however, neither system can destroy the cells of the implant when the pore size of the membrane is properly selected for the application. For example, if allografts are destroyed by immune cells, then the membrane pores must only prevent entry of these cells and thus may be about 0.4 μm or smaller. Likewise, if it is necessary to prevent antibodies from reaching the cells, the pores must be smaller than the smallest of the human antibodies, IgG, which is 150 kDa. Of course, a pore size having a molecular weight cutoff of about 150 kDa or less is suitable for tolerization in both allografts and xenografts.

The use of a permselective membrane prevents the immune system from destroying cells encapsulated therein, even though the immune system recognizes the implant tissue as foreign and mounts a classical response. The immune response cannot destroy the cells because they are protected within the membrane device. Because the immune system cannot destroy the cells even over time, the system will come to tolerate the implant and cease trying to destroy it. While the mechanism for this tolerization is not known, it is analogous to desensitizing patients to allergic immune reactions (i.e. antibiotics or bee stings). In fact, an alternative method to the single tolerizing implant is the addition of more cells with more implants over time if necessary. At this point, the immune system basically recognizes this cellular material as "self" and no longer mounts an immune response against it.

Implantation of full curative dose

When the patient has been tolerized to the cells of the implant, a full curative curative size dose of the tissue or whole organ is administered as described in the following examples.

Whole organ transplants - allografts

In one embodiment, the method is used for a human allograft. In this embodiment, the tissue for the initial implant is taken from a living related kidney donor by biopsy or similar method and a tolerizing dose is implanted into the patient. When the patient is tolerized, the whole kidney is taken from the donor and transplanted into the recipient. The graft is accepted with no continuous immunosuppression being necessary.

Whole organ transplants - xenografts

For most embodiments, it is preferable to use animal organs for human transplants. In these embodiments, the procedure is as follows: suitable animal donors are identified. Sources of these donors may be genetically identical (inbred). Tolerizing cells are taken from any animal in the colony. Later, the whole organ is taken from any other animal in the colony. It is preferable that these sources are free of all contaminants of risk to humans so they would preferably be specific pathogens free (SPF) or gnotobiotic (totally isolated in sterile conditions) colonies or herds. Heart, lungs, livers, kidneys, pancreases and other organs may be used in this

embodiment, thus eliminating the critical shortage of these organs from the limited number of available human organ donors.

Cellular transplants - allografts

In this embodiment, the method is used for human to human cellular transplants. A full size therapeutic dose is obtained from the cadaver donor source as previously described. For example, islet cells are obtained from the pancreas of a human donor. The small amount needed for the tolerizing implant is taken from the preparation, encapsulated and implanted as previously described. The remainder of the cells are cryopreserved by known methods (Kneteman et al, *Transplant. Proc.* 18:182-185, 1986) and are held until tolerization is completed. The full preparation is then thawed and ready for implantation. If, in this embodiment, it is necessary to acquire cells from more than one donor to have enough for a curative implant, then the cells for the initial implant are taken from multiple donors and mixed for the implant. The recipient is therefore tolerized to all of the cells from the multiple donors.

Cellular transplants - xenografts

As with whole organs, the present method allows the use of cellular transplants from animals as well. Cells for the initial implant are taken from genetically identical animals or multiple pooled animals as previously described. When the individual is ready for the full transplant, cells may be taken from any other member of the genetically identical colony or from multiple pooled animals if necessary for sufficient curative quantities.

The implantation procedure for the fully curative dose of cells, whether allograft or xenograft, is dependent on the disease, the quantity of cells, the site, and other factors. For example, for diabetes, a preferred procedure for the implantation of islet cells in humans is to inject the cells through the portal vein so that they become lodged in lobes of the liver. This procedure is done under local anesthesia and is minimally invasive to the patient. For treatment of neural disorders, cells can be implanted into any selected area of the brain by well known stereotaxic surgical procedures. Those skilled in the art will know preferred methods for cellular implantation for each embodiment.

Implants for Prevention of Diseases

Identification of patient populations is dependent on the ability to diagnose patients at high risk of developing certain diseases or those in early stages of the disease. Rapid progress has been made in this area of medicine primarily due to major advances in understanding and mapping the human genome. In addition, DNA amplification methods, notably the polymerase chain reaction (PCR), can be used to diagnose certain genetic disorders. Other research areas for predicting diseases are advancing as well.

In diabetes, the use of immune marker autoantibodies to establish preclinical diabetes has been well studied (Palmer, *Diabetes Rev.* 1(1):104-116, 1993). When these patients are identified, the physician determines at what point in the course of the disease it would be most advantageous to intervene.

Individuals determined to be at risk for development of a particular disease are implanted with the appropriate cell type as described above. Methods for acquiring small amount of cellular tissue for the initial tolerizing implant, tissue types, the amount of tissue necessary for implantation, preparation of the encapsulation device, loading cells into the device, implanting the device into a patient, membrane parameters, device configuration, implantation methods, curative dose administration, etc. are the same as discussed hereinabove for disease treatment.

Treatment of Diseases Arising from Lack of a Hormone

A study was performed using an insulin-producing mouse tumor cell line encapsulated in a permselective membrane coating as described in the following example.

Example 1

Implantation of mouse insulinoma cells

The NIT insulin-producing mouse tumor cell line was encapsulated with PEG conformal coatings of a single configuration, 11% PEG 4,000 kDa molecular weight (See U.S. Patent No. 5,529,914), which corresponds to a molecular weight cutoff of between about 10 kDa and about 70 kDa. The encapsulated cells were implanted beneath the kidney capsule at two different doses into C57B6 mice of a different allograft haplotype in which diabetes had been induced by intravenous injection (tail

vein) of 167 mg/kg body weight of streptozotocin (Upjohn, Kalamazoo, MI). Induction of diabetes by streptozotocin injection is a well known procedure which destroys pancreatic insulin-producing β cells.

Tolerizing doses of encapsulated insulinoma cells were 50 or 100 cell aggregates, each containing about 1,000 cells. Encapsulated cells were implanted beneath the kidney capsule using standard surgical procedures. Curative implants of unencapsulated insulinoma cells (2,000 - 3,000 insulinoma cell aggregates, each containing about 1,000 cells) were administered by free intraperitoneal injection 15 or 20 days after the tolerizing dose to determine whether a sufficient quantity of cells survived. Control animals were given only the curative dose of insulinoma cells. Blood glucose levels were monitored and are shown for the 100 encapsulated NIT aggregate tolerizing dose, 50 encapsulated NIT aggregate tolerizing dose and non-tolerized controls (Figures 5, 6 and 7, respectively).

The severity of streptozotocin-induced diabetes in these mice caused several of the animals to die during the periods of observation and during procedures done as part of the study. Table 1 indicates the number of animals involved in the study and their outcomes. The degree of diabetes is very high, with values over 500 mg/dl (shown as 500) for all streptozotocin-induced animals in the study. Many of these severely diabetic animals died of their diabetes during the study or following a procedure as noted. As shown in Figure 5, of the first group of 8 diabetic mice receiving 100 encapsulated aggregates, only four survived for the challenge 20 days later with the unencapsulated aggregates. Two of these died overnight following the IP injection. The remaining two recipients both had a sudden and marked reduction in their glucose values between 5 and 9 days, with glucose values reaching levels of 40 mg/dl and below (BM5 and BM11, Figure 5 and Table 1). If the insulin-secreting insulinoma cells induce immunological tolerance, the curative implant will be recognized as "self" and will not be destroyed by the recipient's immune system. Because the NIT cells are tumor cells which double every 2-3 days *in vitro*, their survival would be expected to result in recipient hypoglycemia due to the increasing insulin-producing cell mass that would occur from living and growing tumor cells.

In the second group of three recipients of 50 encapsulated aggregates for 15 days, two died of their diabetes prior to challenge with unencapsulated NIT cells. The one animal that received the challenge of unencapsulated NIT cells (BM16) has not exhibited any reduction in blood glucose values for the same time of observation (Figure 6). None of the control animals only challenged with unencapsulated NIT cells exhibited any reduction in blood glucose values (Figure 7).

The results indicate that encapsulated NIT cells given as a small mass prior to a large, unencapsulated curative cell implant permits the second curative dose to survive, reducing blood glucose values in a pattern suggestive of NIT tumor cell growth. A smaller dose of encapsulated NIT cells did not give this result. Control animals that only received unencapsulated NIT cells in a curative dose exhibited no reduction in blood glucose. These results indicate that the preliminary encapsulated implant tolerized the host to the following unencapsulated curative dose. When such a preliminary encapsulated implant was not done, the curative unencapsulated implants had no effect on blood glucose and were presumably destroyed by the host.

Table 1

Animal #	Toler. Encap. Implant	# Encap. Cell Agg. Tol. Dose	Delay to Cure Implant	Unencap. Cell Implant	# Unenc. Cell Agg. Cure	Effect on Blood Glucose
BM1	yes	100	20 days	yes	2348	none-died*
BM3	yes	100	20 days	no-died	-	n/a
BM4	yes	100	19 days	no-died	-	n/a
BM5	yes	100	20 days	yes	2348	down to 40
BM6	yes	100	20 days	no-died	-	n/a
BM7	yes	100	20 days	yes	2348	none-died*
BM10	yes	100	20 days	no-died	-	n/a
BM11	yes	100	19 days	yes	2348	down to 40
BM14	yes	50	15 days	no-died	-	n/a
BM15	yes	50	15 days	no-died	-	n/a
BM16	yes	50	15 days	yes	2348	none-500

Animal #	Toler. Encap. Implant	# Encap. Cell Agg. Tol. Dose	Delay to Cure Implant	Unencap. Cell Implant	# Unenc. Cell Agg. Cure	Effect on Blood Glucose
BM29	no	0	-	yes	2352	none-500
BM31	no	0	-	yes	2352	none-500
BM32	no	0	-	yes	2352	none-500
BM34	no	0	-	yes	2352	none-500
BM35	no	0	-	yes	2352	none-died*
BM36	no	0	-	yes	2352	none-500
BM40	no	0	-	yes	2352	none-500
BM41	no	0	-	yes	2352	none-died*
BM42	no	0	-	yes	2352	none-500
BM43	no	0	-	yes	2352	none-died*
BM44	no	0	-	yes	2352	none-500
BM45	no	0	-	yes	2352	none-500

* Died during course of experiment-no effect on blood glucose

Example 2

Use of encapsulated islets for induction of allograft tolerance in rats

Rat pancreatic islet cells are isolated by a standard collagenase digestion method (Ricordi, *Diabetes* 37:413-410, 1988) and cultured for three days prior to PEG encapsulation. Donor islets are derived from the Wistar Furth (WF) strain having MHC haplotype RT1-U. Recipients are of the Lewis strain having MHC haplotype RT1-1. Transplants across this strain combination are normally rejected within three weeks. Islet transplant mass is dosed on the basis of a standard 150 μm diameter rat islet; an Islet Equivalent (Ieq). Islets are quantified and tested for sterility and mycoplasma prior to encapsulation and implantation.

Islet cells are conformally coated with 11% PEG 4,000 kDa molecular weight by the method described in U.S. patent No. 5,529,914. As a negative control, acellular cross-linked dextran beads are encapsulated in a similar manner. Diabetes is induced in fasted Lewis rats by intravenous injection of streptozotocin (65 mg/kg) one week prior to implantation of the tolerizing dose and monitored during that week for blood glucose levels and weight changes. Rats are considered diabetic once their

blood glucose level exceeds 350 mg/dl. Rats having a minimal weight loss and blood glucose levels of 300-350 mg/dl are used for the study.

Diabetic rats are implanted by trochar with a subcutaneous 30 day time release depot insulin (Linplant, Lishin, Ontario, Canada) to reduce the chances of ketosis/acidosis and to stabilize their diabetes. Animals remain hyperglycemic at this Linplant dose (2 units of bovine insulin in 24 hours - lasts 30 days).

Diabetic MHC disparate Lewis rats are surgically implanted once with encapsulated donor WF islets at the renal subcapsular site after anesthetization. The dose of implanted cells varies as outlined in Table 2.

Table 2

Group	N	Dose	Rationale
1	12	1200 encap islets	high dose sensitization/tolerization
2	12	600 encap islets	low dose tolerization
3	12	300 encap islets	very low dose tolerization
4	12	1200 encap acellular beads	control for polymer

As a control, a set of recipients (Group 4) is implanted with encapsulated acellular beads to control for possible polymer effects in tolerization. All implanted animals are maintained for intervals as shown in Table 3 prior to the second transplantation. At the time of implantation, serum samples from each animal are drawn and retained for future immunological analysis.

Table 3

Group	N	Dose	Implant Interval (days)	Rationale
1a	4	1200 encap islets	30	high dose sensitize/tolerize-short interval
1b	4	1200 encap islets	60	high dose sensitize/tolerize-intermediate interval
1c	4	1200 encap islets	90	high dose sensitize/tolerize-long interval
2a	4	600 encap islets	30	low dose tolerization-short interval
2b	4	600 encap islets	60	low dose tolerization-intermediate interval
2c	4	600 encap islets	90	low dose tolerization-long interval
3a	4	300 encap islets	30	very low dose tolerization-short interval
3b	4	300 encap islets	60	very low dose tolerization-intermediate interval
3c	4	300 encap islets	90	very low dose tolerization-long interval

4a	4	1200 encap acell-beads	30	polymer control-short interval
4b	4	1200 encap acell-beads	60	polymer control-intermediate interval
4c	4	1200 encap acell-beads	90	polymer control-long interval

During the indicated period, animals are monitored for weight changes and blood glucose levels. One week before the second transplant, one animal in each of Groups 1a-1c, 2a-2c, 3a-3c and 4a-4c is sacrificed and the implant site analyzed by histological methods for determining viability of the tolerizing cells.

Lewis rats remaining in Groups 1-4 receive a second transplant (curative dose) of WF islets which are unencapsulated. Transplant sites in each animal are intraportal (IP) at a dose of 6,000 Ieq and at one kidney with a dose of 100 Ieq (See Table 4). 6,000 Ieq implanted into the liver is known to be a curative dose in the rat diabetes model. The 100 Ieq kidney capsule implant is only for histology at the end of the experiment. At the time of the second implant, serum samples from each animal are drawn and retained for future immunological analysis. For the next three weeks, animals are monitored for blood glucose levels and weight changes. At the termination of the experiment, graft sites are processed for histology. At this time, serum samples from each animal are again drawn and retained for future immunological analysis.

Table 4

Group	N	Recipient haplotype	Dose # of islets	Implant Sites	Duration of Transplant
1a	3	RT1-1	6000	IP/kidney	3 weeks
1b	3	RT1-1	6000	IP/kidney	3 weeks
1c	3	RT1-1	6000	IP/kidney	3 weeks
2a	3	RT1-1	6000	IP/kidney	3 weeks
2b	3	RT1-1	6000	IP/kidney	3 weeks
2c	3	RT1-1	6000	IP/kidney	3 weeks
3a	3	RT1-1	6000	IP/kidney	3 weeks
3b	3	RT1-1	6000	IP/kidney	3 weeks
3c	3	RT1-1	6000	IP/kidney	3 weeks

4a	3	RT1-1	6000	IP/kidney	3 weeks
4b	3	RT1-1	6000	IP/kidney	3 weeks
4c	3	RT1-1	6000	IP/kidney	3 weeks

In Groups 1 and 4, no changes in the diabetic state are measured. In Group 4, rejection occurs in the expected two week time frame as measured by a transient normoglycemia followed by a return to the diabetic state. In Group I, a more rapid rejection of the implant due to sensitization of the recipients occurs. In the recipients previously exposed to tolerizing doses of encapsulated WF islets (Groups 2 and 3), islet cells survive and result in a continuous maintenance of normoglycemia.

Example 3

Use of encapsulated islets for induction of allograft tolerance in humans

Human islets are isolated from cadavers and 1,500 islets/kg body weight are PEG-encapsulated and implanted under the kidney capsule in a diabetic patient. After two months, a curative dose of 15,000 unencapsulated islets/kg body weight are injected intraportally. Insulin administration is continued during the course of the protocol up to administration of the curative dose. Blood glucose levels are constantly monitored and are within the normal range.

Example 4

Treatment of Parkinson's disease (xenograft)

Adrenal chromaffin cells are isolated from inbred baboon adrenal glands and 1,000 cells/kg body weight are encapsulated in an appropriate PEG conformal coating. The capsule is implanted into the interstitial brain region of a human by a neurosurgeon using stereotaxic instruments. After 1 month of tolerization, 10,000 unencapsulated cells/kg body weight are injected into the same brain region. Significant improvement in the condition is observed.

Example 5

Prevention of hemophilia

A male individual at risk of developing hemophilia, an x-linked disorder, by virtue of family history, is subjected to genetic screening to determine the presence or absence of the gene encoding Factor VIII, and to clotting time analysis. If the gene

is absent or clotting time is reduced, 2,500 liver cells/kg recipient body weight are isolated from a human donor and encapsulated in a PEG conformal coating. The encapsulated cells are implanted under the kidney capsule. One month later, 5,000 cryopreserved liver cells/kg recipient body weight (from the same donor) are injected intraportally. Clotting time is significantly improved.

Example 6

Liver transplant (xenograft)

An individual in need of a liver transplant is subcutaneously implanted with 1,000 PEG-encapsulated liver cells/kg body weight isolated from an inbred baboon. Two months later, the entire liver is transplanted into the individual. Signs of organ rejection and vital signs are monitored over several months. Rejection does not occur.

Example 7

Prevention of myasthenia gravis (xenograft)

Myasthenia gravis is an autoimmune disorder resulting from the presence of antibodies against the acetylcholine receptor on neurons. An individual having very early signs of the disease is implanted under the kidney capsule with a tolerizing dose of 2,500 PEG-encapsulated neural cells/kg recipient body weight expressing the acetylcholine receptor isolated from baboons. This results in tolerization to the acetylcholine receptor and prevention of the disorder.

It should be noted that the present invention is not limited to only those embodiments described in the Detailed Description. Any embodiment which retains the spirit of the present invention should be considered to be within its scope. However, the invention is only limited by the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method of treating diabetes in a mammal in need thereof, comprising the steps of:

implanting in said mammal a tolerizing dose of insulin-secreting cells from the same species as said mammal encapsulated in a biologically compatible permselective membrane; then

administering to said mammal a curative dose of corresponding unencapsulated insulin-secreting cells.

2. The method of claim 1, wherein said mammal is a human, canine or feline.

3. The method of claim 1, wherein said tolerizing dose is one to two orders of magnitude less than said curative dose.

4. The method of claim 1, wherein said insulin-secreting cells are pancreatic islet cells.

5. The method of claim 1, wherein said membrane comprises polyethylene glycol.

6. The method of claim 1, wherein said tolerizing and curative doses are porcine.

7. The method of claim 1, further comprising the step of administering one or more anti-inflammatory agents to said mammal prior to, at the same time as, or subsequent to administration of said curative dose.

8. The method of claim 1, wherein said membrane has a molecular weight cutoff of about 150 kDa or less.

9. The method of claim 1, wherein said membrane has a pore size of less than about 0.4 μm.

10. The method of Claim 9, wherein said membrane has a pore size of less than about 0.2 μm.

11. The method of Claim 1, wherein said curative dose is between one and two orders of magniture higher that said tolerizing dose.

12. The method of Claim 1, wherein said implanting step is subcapsular, subcutaneous, intraperitoneal or intraportal.

13. The method of Claim 1, wherein said administering step is intraperitoneal, intraportal or subcutaneous.

14. The method of Claim 1, wherein said tolerizing dose is administered incrementally.

INDUCTION OF IMMUNOLOGICAL TOLERANCE

Abstract of the Invention

A method of creating tolerance to transplanted cells, tissue, or organs without the need for continuous immunosuppression. A tolerizing dose of a cell or tissue within a membrane structure is implanted into a patient. Once the patient becomes tolerant to the cell or tissue, a tissue or organ is implanted which will no longer be recognized as foreign matter. The method makes animal organs practical for human use, prevents autoimmune destruction as well as immune rejection. It has applications in treatment and prevention of many mammalian diseases.

NSB-3787
102496

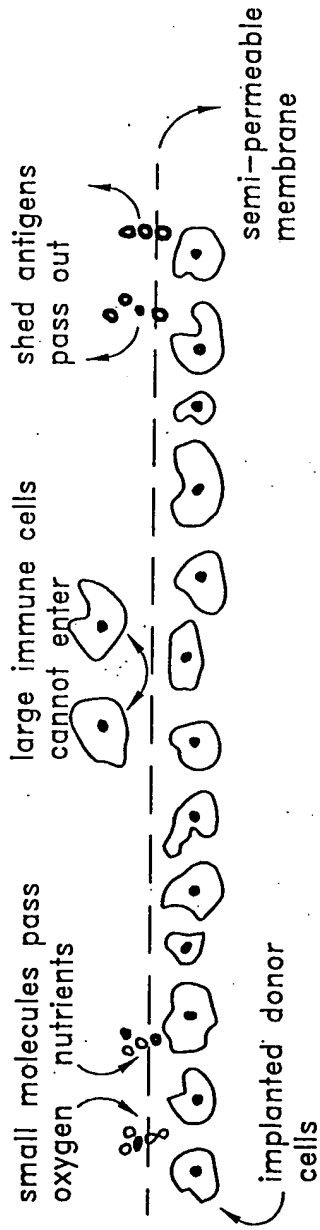
small molecules pass
oxygen nutrients
large immune cells
cannot enter
shed antigens
pass out
semi-permeable
membrane
implanted donor
cells

FIG. 1

FIG. 2

FIG. 3

FIG. 4

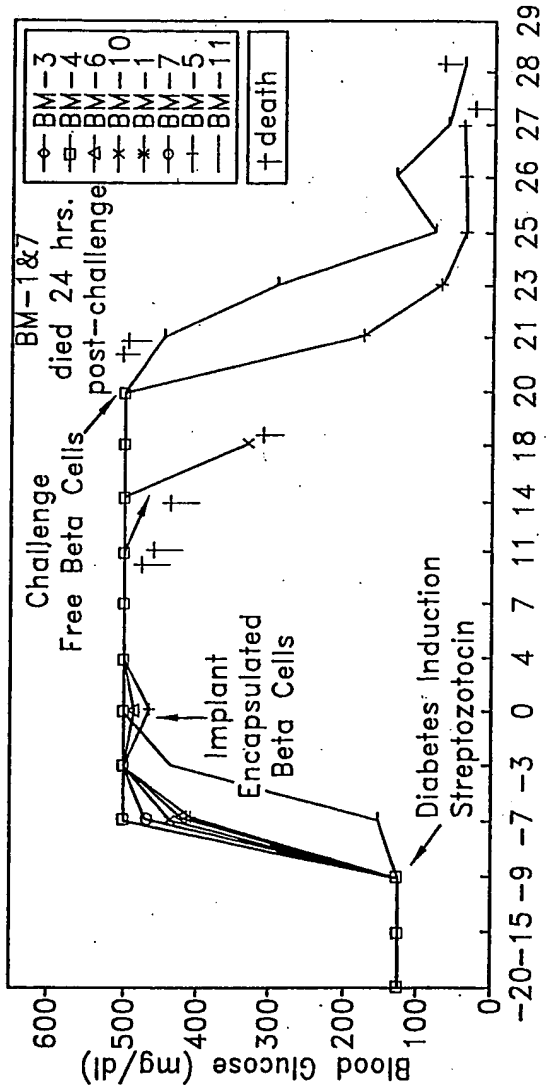
BM-3
BM-4
BM-6
BM-10
BM-1
BM-7
BM-5
BM-11
†death
BM-1&7 died 24 hrs. post-challenge
Challenge Free Beta Cells
Implant Encapsulated Beta Cells
Diabetes Induction Streptozotocin
Blood Glucose (mg/dl)
600
500
400
300
200
100
0
-20 -15 -9 -7 -3 0 4 7 11 14 18 20 21 23 25 26 27 28 29
Days Post Capsule Implantation

FIG. 5

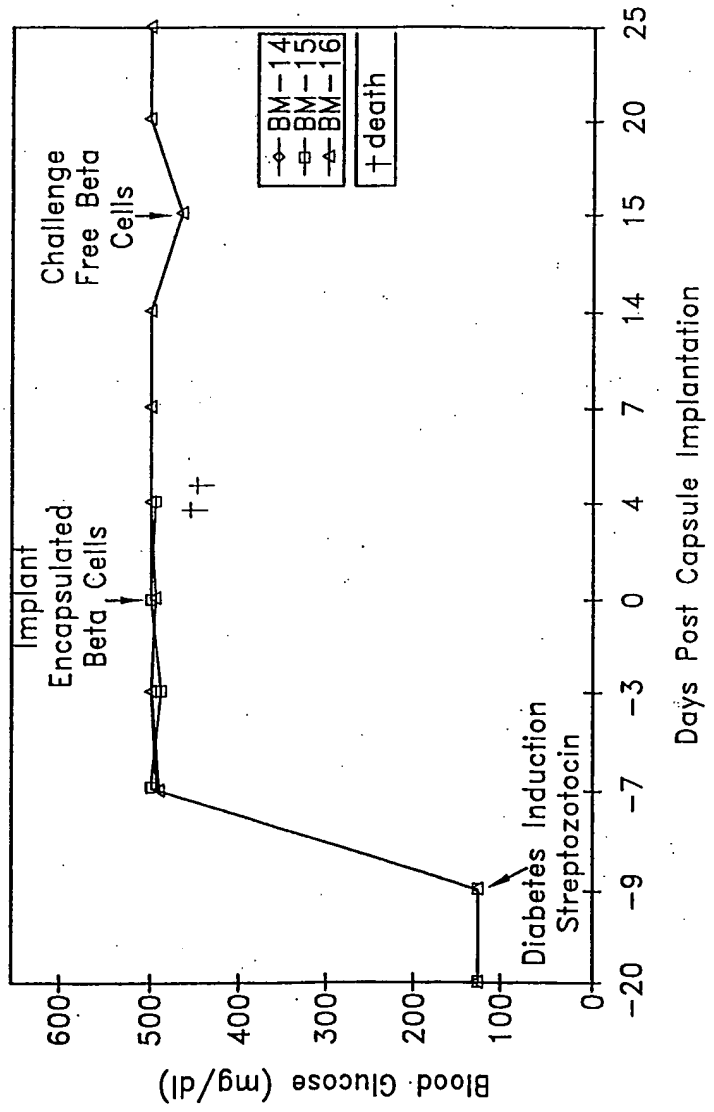
Blood Glucose (mg/dl)
600
500
400
300
200
100
0
-20
-9
-7
-3
0
4
7
14
15
20
25
Days Post Capsule Implantation
Diabetes Induction Streptozotocin
Implant Encapsulated Beta Cells
Challenge Free Beta Cells
BM-14
BM-15
BM-16
†death
††

FIG. 6

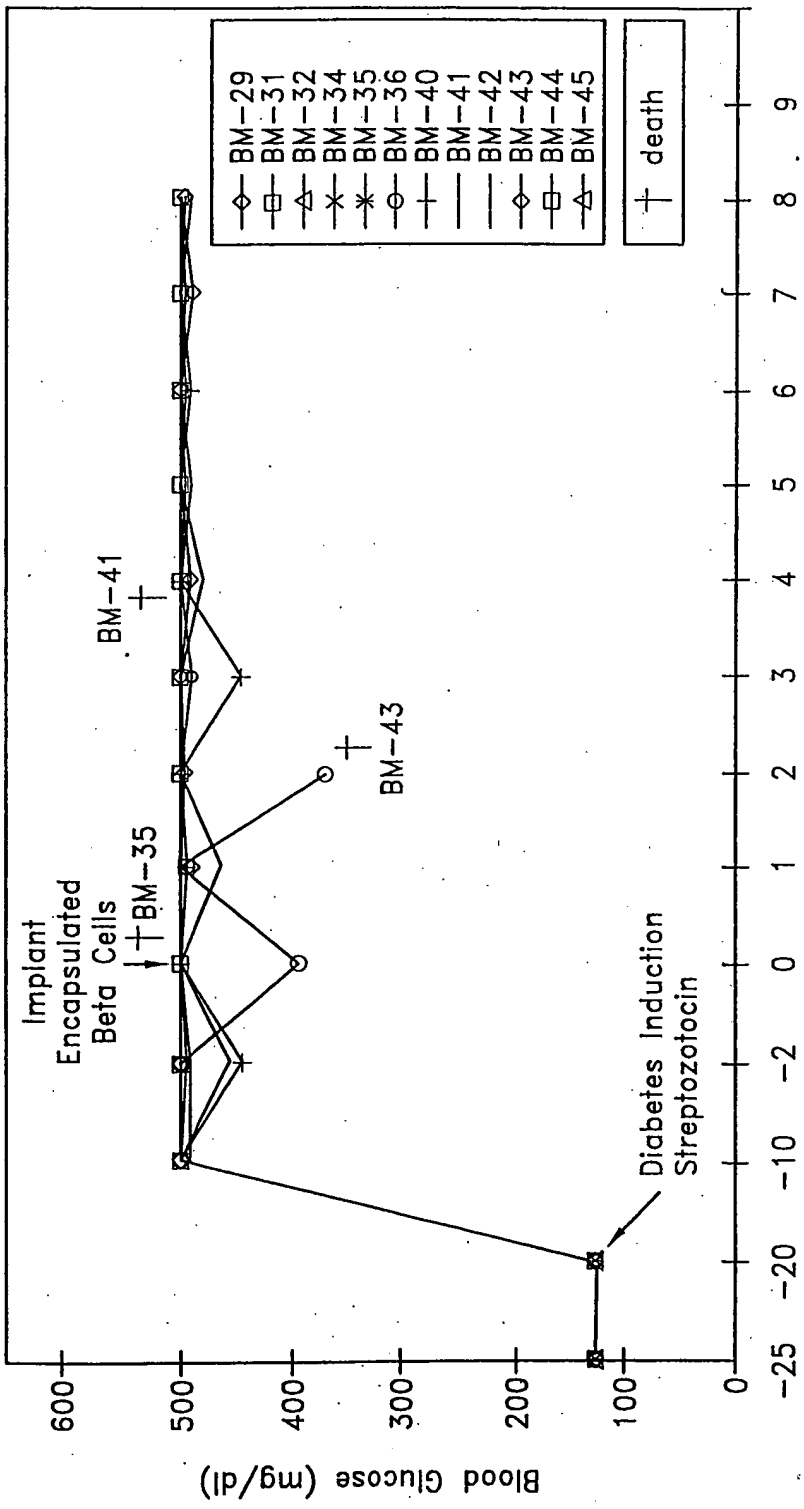
Blood Glucose (mg/dl)
600
500
400
300
200
100
0
-25
-20
-10
-2
0
1
2
3
4
5
6
7
8
9
Days Post Capsule Implantation
BM-29
BM-31
BM-32
BM-34
BM-35
BM-36
BM-40
BM-41
BM-42
BM-43
BM-44
BM-45
death
Implant Encapsulated Beta Cells
BM-35
BM-41
BM-43
Diabetes Induction Streptozotocin

FIG. 7

LATTA.002C4 MX
DEA/MXG

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ART UNIT	PAPER NUMBER
1644	

DATE MAILED: 01/26/2005

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	10/823,263	LATTA, PAUL P.	
	Examiner	Art Unit	
	Michail A Belyavskyi	1644	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *12 October 2004*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-14* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-14* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

1. Claims 1-14 are pending.

2. Applicant's election without traverses of human as spices of mammal , intraportal as species of implanting step in the reply filed on 10/12/04 is acknowledged.

Claims 1-14 read on a method of treating diabetes in mammal, wherein mammal is human, comprising implanting a tolerizing dose of insulin-secreting cells encapsulated in a biologically compatible permselective membrane and then administering a curative dose of corresponding unencapsulated insulin-secreting cells, wherein administering is intaportal under consideration in the instant application.

3. The title of the invention is not descriptive. A new title is required that is clearly indicative of the invention *to which the claims are directed.*

4. Applicant notes that an IDS was submitted with the prior application 10/660,924. However these citations have been crossed out as said references cited in said parent application cannot be found. Applicant is invited to resubmit such references to complete the instant file. The examiner apologizes for any inconvenience to applicant for having to resubmit such documents.

5. The following is a quotation of the second paragraph of 35 U.S.C. 112.
The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

6. Claims 1-14 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

A. Claims 1, 6 and 11 recites the limitation "administering a curative dose" There is insufficient antecedent basis for this limitation in the claim. The preamble of the base claim 1 recites " a method of treating diabetes", not a method of curing diabetes.

B. Claim 6 is indefinite and ambiguous in the recitation of "..wherein said tolerizing and curative doses are porcine". It is unclear what Applicant means by this phrase, since " doses" can not be porcine.

7. The following is a quotation of the first paragraph of 35 U.S.C. 112:
The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

8. Claims 1-14 are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and or use the invention commensurate in scope with this claim.

The specification disclosure does not enable one skilled in the art to practice the invention without an undue amount of experimentation.

Factors to be considered in determining whether undue experimentation is required to practice the claimed invention are summarized *In re Wands* (858 F2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988)). The factors most relevant to this rejection are the scope of the claim, the amount of direction or guidance provided, the limited working examples, the unpredictability in the art and the amount of experimentation required to enable one of skill in the art to practice the claimed invention.

The specification only discloses the effects of the implanting of insulin-producing cells on the level of blood glucose using streptozotocin-induced diabeties in murine experimental model. (See Examples 1-2 in particular). Examples 3-7 in the instant Specification are prophetic examples that indicate what the inventor thinks might happen in the experiments which have not actually been performed. The specification does not adequately teach how to effectively treat diabetes in mammal , wherein mammal is human, comprising implanting a tolerizing dose of insulin-secreting cells encapsulated in a biologically compatible permselective membrane and then administering a curative dose of corresponding unencapsulated insulin-secreting cells wherein administrating step is intaportal . Moreover, the specification does not teach whether administering of second i.e. curative dose that is one or two orders of magnitude more than tolerizing dose will be tolerated (i.e. not rejected) in human. Roep et al (Nature Reviews, 2004, v.4, pages 989-997) teaches that despite more than two decades of productive research, we are still yet to define an initiating autoantigen for human disease, to determinethe precise mechanism of β-cell destruction in human and to design invention thatprevent or cure type I diabetes. Studying the pathogenesis of diabetes in human is difficult. No animal model had

been developed that shows a reproducibly high incidence of disease in a timely experimental manner. Many of the preventive therapies that seems to be promising in mouse models did not show similar efficacy in human patient. We believed that it is necessary to re-evaluate and consider at a higher level the apparent difference in the immunology and biology of diabetes between mice and men. (see entire documewnt, Abstract, pages 989, 990 and Table 1 in particular). Mestas et al (J. of Immunology, 2004, 172, pages 2731-238) teach that there exist significant differences between mice and humans in immune system development, activating and response to challenge in both the innate and adaptive arms. As therapies for human diseases become ever more sophisticated and specifically targeted it becomes increasing important to understand the potential limitations of extrapolating data from mice to humans. The literature is littered with the examples of therapies that work well in mice but fail to provide similar efficacy in humans. Teuveson et al., (Immun. Review 1993, N136, pages 101-107) teach that one problem with rodent models of transplantation is that rejection is easily overcome in said models in comparison to the difficulty of overcoming allograft rejection in human (see page 100 in particular). Teuveson et al., further teach that " however today's small animal models seem to be insufficient to produce data for clinical decision-making" and further raises doubt as to whether large animal models can be applied to clinical situations, due to species-specific reactions to treatment (see page 101 in particular). Feldman et al (Transplant. Proc. 1998, 30, 4126-4127) teach that "while it is not difficult to study the pathogenesis of animal models of disease, there are multiple constraints on analyses of the pathogenesis of human disease, leading to interesting dilemmas such as how much can we rely on and extrapolate from animal models in disease". Moreover, since the a method of treating diabetes in mammal, wherein mammal is human, comprising implanting a tolerizing dose of insulin-secreting cells encapsulated in a biologically compatible permselective membrane and then administering a curative dose of corresponding unencapsulated insulin-secreting cells can be species- and model-dependent (see Van Noort et al. International Review of Cytology, 1998, v.178, pages 127-204, Table III in particular) , it is not clear that reliance on the *in vivo* murine data accurately reflects the relative any mammal and human efficacy of the claimed therapeutic strategy. Van Noort et al., further indicate factors that effect immune response such as genetic, environmental and hormonal (Page 176, Paragraph 3). The ability of a host to enhance an immune response will vary depending upon factors such as the condition of the host and burden of disease.

Thus, as has been discussed supra, the state of the art is that it is unpredictable form the *in vivo* murine data disclosed in the specification as whether the instant invention can be used for the *in vivo* treatment of diabetes in any mammals including human. Therefore, it is not clear that the skilled artisan could predict the efficacy a method of treating diabetes in mammal, wherein mammal is human, comprising implanting a tolerizing dose of insulin-secreting cells encapsulated in a biologically compatible permselective membrane and then administering a curative dose of corresponding unencapsulated insulin-secreting cells. Thus in the absence of working examples or detailed guidance in the specification, the intended uses of the claimed method of treating diabetes in mammal, wherein mammal is human, comprising implanting a tolerizing dose of insulin-secreting cells encapsulated in a biologically compatible permselective membrane and then administering a curative dose of corresponding unencapsulated insulin-secreting cells are fraught with uncertainties.

Thus, Applicant has not provided sufficient guidance to enable one skill in the art to use claimed a method of treating diabetes in mammal, wherein mammal is human, comprising implanting a tolerizing dose of insulin-secreting cells encapsulated in a biologically compatible permselective membrane and then administering a curative dose of corresponding unencapsulated insulin-secreting cells in manner reasonably correlated with the scope of the claims. The scope of the claims must bear a reasonable correlation with the scope of enablement. *In re Fisher*, 166 USPQ 18(CCPA 1970) indicates that the more unpredictable an area is, the more specific enablement is necessary in order to satisfy the statute.

In view of the quantity of experimentation necessary, the unpredictability of the art, the lack of sufficient guidance in the specification, the limited working examples, and the limited amount of direction provided given the breadth of the claims, it would take undue trials and errors to practice the claimed invention.

9. The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

10. Claims 1-4 and 6 -14 are rejected under 35 U.S.C. 103(a) as being unpatentable over US Patent 6,703,017 or by US Patent 5,425764 or US Patent 5,629,194 each in view Posselt et al (Diabetes, 1992, v.41, pages 771-775) as is evidenced by the disclosure of Specification on overlapping pages 12-13.

US Patent '017 teaches a method of treating diabetes in a mammal comprising implanting insulin-producing cells encapsulated in a biologically compatible membrane (see entire document, Abstract and columns 6, 8, 9 –14 and Example 12 in particular) . US Patent '017 teaches that insulin producing cells are pancreatic islet cells from primary cell source (see columns 8 and 11 in particular). US Patent '017 teaches that pancreatic islet cells are from the

same species as the mammal and are implanted interperitoneally into the tissue of a mammal beneath the kidney capsule (see overlapping columns 13-14 and Example 2 in particular). US Patent '017 teaches that encapsulation of said insulin-producing cells in biologically compatible membrane for success of implantation is well known in the art (see column 12 and Example 12 in particular).

US Patent '764 teaches a method of treating diabetes in a mammal comprising implanting insulin-producing cells encapsulated in a biologically compatible membrane (see entire document, Abstract and overlapping columns 5-6 in particular). US Patent '764 teaches that insulin producing cells are pancreatic islet cells (see column 1 and 4 in particular). US Patent '764 teaches that cells are implanted interperitoneally (see column 5 in particular).

US Patent '194 teaches a method of treating diabetes in mammal comprising implanting insulin-producing cells encapsulated in a biologically compatible membrane (see entire document, Abstract overlapping columns 7-8 , 12 and Example II in particular). US Patent '764 teaches that insulin producing cells are pancreatic islet cells (see column 8 in particular). US Patent '764 teaches that cells are implanted intaportal (see column 7 in particular). US Patent '194 teaches administration of one or more anti-inflammatory agent at the dosage sufficient to achieve the desired therapeutic effect. US Patent '194 teaches that said agent can be administered prior to at the same time or subsequent to administration of insulin-producing cells (see overlapping columns 14-15 in particular).

US Patent '017 or US Patent ' 764 or US Patent '194 does not explicitly teaches a method of treating diabetes in a mammal comprising administration two doses of insulin-secreting cells one tolerizing and one curative wherein tolerizing doze is one order less than curative.

Posselet et al., teach that the important goal in the treatment of insulin-dependent diabetes by pancreatic islet transplantation is the development of strategies that allow permanent survival of pancreatic islet without continuous host immunosuppression. Posselet et al., further teach a strategy comprising two step process : first administering a small dose of cells that induces an unresponsive state, i.e. tolerizing dose and then administering fully therapeutic dose, at another site (see entire document, Abstract in particular). Posselet et al., teach that said strategy permits the survival of pancreatic islet transplant.

The Specification on overlapping pages 12-13 disclosed that curative dose is fully therapeutic dose.

It would have been obvious to a person of ordinary skill in the art at the time the invention was made to apply the teaching of Posselt at al. to those of US Patent '017 or US Patent ' 764 or US Patent '194 to obtain a claimed method of treating diabetes in a mammal comprising

administration two doses of insulin-secreting cells one tolerizing and one curative wherein tolerizing doze is one order less than curative

One of ordinary skill in the art at the time the invention was made would have been motivated to do so, because a strategy comprising two step process : first administering a small dose of cells that induces an unresponsive state, i.e. tolerizing dose and then administering fully therapeutic dose, at another site permits the survival of pancreatic islet transplant as taught by Posselet et al. Said strategy can used in the method of treating diabetes in a mammal, comprising implanting pancreatic islet, taught by US Patent '017 or US Patent ' 764 or US Patent '194. The strongest rationale for combining references is a recognition, expressly or impliedly in the prior art or drawn from a convincing line of reasoning based on established scientific principles or legal precedent, that some advantage or expected beneficial result would have been produced by their combination. In re Semaker. 217 USPQ 1, 5 - 6 (Fed. Cir. 1983). See MPEP 2144.

From the combined teaching of the references, it is apparent that one of ordinary skill in the art would have had a reasonable expectation of success in producing the claimed invention. Therefore, the invention as a whole was *prima facie* obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references, especially in the absence of evidence to the contrary.

Claims 8-14 are included because it would be conventional and within the skill of the art to : (i) determine the proper pore size for the permselective membrane or (ii) to determine the optimum dosage and means of administration of insulin-secreting cells in an absent of a showing of unobvious property. Moreover, Applicant acknowledge that one of ordinary skill in the art can readily determine the proper pore size for the permselective membrane (see page 8, line 13-20 of the instant Specification in particular). Further, it has been held that where the general conditions of a claim are disclosed in the prior art, discovering the optimum or workable ranges or means of administration involves only routine skill in the art. *In re Aller*, 220 F2d 454,456,105 USPQ 233; 235 (CCPA 1955). see MPEP § 2144.05 part II A.

11. Claim 5 is rejected under 35 U.S.C. 103(a) as being unpatentable over US Patent 6,703,017 or by US Patent 5,425764 or US Patent 5,629,194 each in view Posselt et al (Diabetes, 1992, v.41, pages 771-775) as applied to claims 1-4 and 6-14 above, and further in view of US Patent 5,529,914.

The teaching of US Patent " 017, US Patent ' 764 , US Patent' 194 and Posselt et al., have been discussed, supra.

The combined references do not explicitly teachers a method of treating diabetes in a mammal comprising implanting insulin-secreting cells, wherein insulin-secreting cells are encapsulated in a biologically compatible membrane wherein said membrane comprises polyethylene glycol (PEG).

US Patent '914 teaches a new type of biocompatible membrane as a covering to encapsulate biological materials, comprising PEG that is acceptable for implants in mammalian. (see entire document, Abstract in particular). US Patent '914 teaches that various types of cells can be encapsulated in said biocompatible membrane and that said encapsulation will prevent rejection of encapsulated cells during transplantation (see column 10 in particular).

It would have been obvious to a person of ordinary skill in the art at the time the invention was made to apply the teaching of US Patent '914 to those of US Patent " 017, US Patent ' 764 , US Patent' 194 and Posselt et al., to obtain a claimed method of treating diabetes in a mammal comprising implanting insulin-secreting cells, wherein insulin-secreting cells are encapsulated in a biologically compatible membrane wherein said membrane comprises polyethylene glycol (PEG).

One of ordinary skill in the art at the time the invention was made would have been motivated to do so, because encapsulation of cells in biologically compatible membrane comprising PEG will prevent rejection of encapsulated cells during transplantation as taught by US Patent '914. Said type of biocompatible membrane can be used to substitute the different type of biocompatible membrane for successful implantation of insulin-producing cells in the method of treating diabetes taught by combined references of US Patent " 017, US Patent ' 764 , US Patent' 194 and Posselt et al. The strongest rationale for combining references is a recognition, expressly or impliedly in the prior art or drawn from a convincing line of reasoning based on established scientific principles or legal precedent, that some advantage or expected beneficial result would have been produced by their combination. In re Semaker. 217 USPQ 1, 5 - 6 (Fed. Cir. 1983). See MPEP 2144.

From the combined teaching of the references, it is apparent that one of ordinary skill in the art would have had a reasonable expectation of success in producing the claimed invention. Therefore, the invention as a whole was *prima facie* obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references, especially in the absence of evidence to the contrary.

12. No claim is allowed.

13. The lengthy specification has not been checked to the extent necessary to determine the presence of all possible minor errors. Applicant's cooperation is requested in correcting any errors of which Applicant may become aware in the specification.

14. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Michail Belyavskyi whose telephone number is 571/272-0840. The examiner can normally be reached Monday through Friday from 9:00 AM to 5:30 PM. A message may be left on the examiner's voice mail service. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Christina Chan can be reached on 571/272-0841.

The fax number for the organization where this application or proceeding is assigned is 703-872-9306.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Michail Belyavskyi, Ph.D.
Patent Examiner
Technology Center 1600
January 24, 2005

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SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600

FORM PTO-1449 U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTY. DOCKET NO. LATTA.002C4	APPLICATION NO. 10/823,263
INFORMATION DISCLOSURE STATEMENT BY APPLICANT	APPLICANT PAUL P. LATTA	
(USE SEVERAL SHEETS IF NECESSARY)	FILING DATE April 13, 2004	GROUP Unknown

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	17.	A1 2,034,641	28/05/1992	CANADA				
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(USE SEVERAL SHEETS IF NECESSARY)	FILING DATE April 13, 2004	GROUP Unknown

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	Examiner Michail A Belyavskyi	Art Unit 1644	Page 1 of 2

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	C	US-5,629,194	05-1997	Dinsmore, Jonathan	435/325
	D	US-5,529,914	06-1996	Hubbell et al.	435/182
	E	US-			
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*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
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NON-PATENT DOCUMENTS

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✓	U	Posselt et al Diabetes, 1992, v.41, pages 771-775
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	W	
	X	

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Promotion of Pancreatic Islet Allograft Survival by Intrathymic Transplantation of Bone Marrow

ANDREW M. POSSELT, JON S. ODORICO, CLYDE F. BARKER, AND ALI NAJI

An important goal in the treatment of insulin-dependent diabetes by pancreatic islet transplantation is the development of strategies that allow permanent survival of islet allografts without continuous host immunosuppression. In this study, we demonstrate that inoculation of allogeneic bone marrow into the thymus of adult rats treated with a single dose of anti-lymphocyte serum induces an unresponsive state that permits survival of subsequent pancreatic islet allografts transplanted to an extrathymic site. This effect is donor specific, cannot be reproduced by systemic administration of bone marrow, and is associated with persistence of chimeric cells in the thymus of the recipient. In addition, lymph node cells from long-term recipients of intrathymic bone marrow display markedly reduced proliferative responses to donor alloantigens in mixed lymphocyte culture. Interaction of maturing thymocytes with foreign alloantigens may produce the unresponsiveness. This model offers a potential approach for establishing donor-specific allograft acceptance in adult recipients. *Diabetes* 41:771–75, 1992

Induction of donor-specific unresponsiveness represents the ideal approach for securing permanent survival of pancreatic islet allografts because it precludes rejection without the need for chronic immunosuppression of the host. Although it was demonstrated >30 yr ago that immunologic tolerance can be readily achieved in rodents by inoculation of donor-strain lymphohematopoietic cells at birth, tolerance induction in adult recipients has been more difficult, requiring extensive preparative conditioning by irradiation and/or treatment with nonspecific cytoablative chemotherapy (1–4). We previously reported that pancreatic islets implanted in the thymus of allogeneic adult rats survived permanently and, in addition, rendered the recipients tolerant of donor alloantigens (5). In this study, the efficacy of this approach in promoting transplantation tolerance was assessed by examining the impact of intrathymic inoculation of allogeneic bone marrow cells (BMC) on the survival of extrathymic transplants of pancreatic islets. We demonstrate that rats pretreated with an intrathymic injection of allogeneic BMC are rendered specifically unresponsive to donor alloantigens and permanently accept subsequent donor-strain islet allografts. Furthermore, this result can be accomplished without need for other methods known to prolong islet allograft survival, such as pretransplant modulation of allograft immunogenicity or chronic immunosuppression of the recipient (6).

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RESEARCH DESIGN AND METHODS

Bone marrow inoculation and islet transplantation. BMC were obtained from adult male Lewis ($RT1^l$) donors, depleted of contaminating erythrocytes by centrifugation on a Ficoll-isopaque gradient and inoculated intravenously into an abdominally displaced testicle or into both thymic lobes of histoincompatible male Wistar Furth (WF) ($RT1^u$) recipients. Each recipient received $60–70 \times 10^6$ nucleated cells; rats given intravenous BMC inocula received intrathymic injections of saline. Where noted, recipients were treated with a single dose of 1 cc rabbit anti-rat lymphocyte serum (ALS) i.p. (Accurate Chemical and Scientific, Westbury, NY) on the day of BMC injection. No additional immunosuppression was administered at any other time during the experiment. The rats were then rendered diabetic with 65 mg/kg streptozocin i.v. and received freshly isolated (uncultured) Lewis or DA ($RT1^a$) islet allografts beneath the renal capsule 14 days after BMC inoculation. Only rats with nonfasting blood glucose levels >300 mg/dl

ELSEVIER

Anti-TNFα Therapy Is Useful in Rheumatoid Arthritis and Crohn's Disease: Analysis of the Mechanism of Action Predicts Utility in Other Diseases

M. Feldman, P. Taylor, E. Paleolog, F.M. Brennan, and R.N. Maini

WHILE it is not to difficult to study the pathogenesis of animal models of disease, there are multiple constraints on analyses of the pathogenesis of human disease, leading to interesting dilemmas such as how much can we rely on and extrapolate from animal models in disease?

In the late 1980s, as a result of investigating cytokine expression and regulation in rheumatoid synovium, both in vitro and in vivo, we found that tumor necrosis factor (TNF)α was of major importance, as it regulated the expression of the other proinflammatory cytokine interleukin-1 (IL-1).[1] We proposed that blocking TNFα would thus have major effects on the inflammatory process and hence would be clinically useful.[2] That prediction has been substantiated in clinical trials that we have led using Centocor's chimaeric monoclonal antibody, cA2, now termed Remicaide. Positive results were also reported using a humanized antibody produced by Celltech[3] and also using a TNF-receptor p75 IgGFc fusion protein, produced by Immunex, termed Enbrel.[4] Clinical effects of anti-TNFα have recently been reviewed.[5]

RESULTS AND DISCUSSION

While the clinical results with the above three biologic therapeutic agents have been in the same ball park, mechanism of action studies reported are only with cA2. The antibodies differ from TNF-R p75Fc in that the latter also blocks the related protein lymphotoxin, while the former are specific to TNFα. The consequences of this difference are not known, but differences are conceivable.

The first pathogenic mechanisms that occur to be clarified in rheumatoid arthritis was the existence of a cytokine cascade in vivo, as had been described in vitro. The effect was rapid diminution of serum IL-6, and other cytokine levels.[5,6] This accounts, in part, for the rapid onset of beneficial effects seen locally. Equally interesting was the observation that levels of cytokine inhibitors such as IL-1ra and soluble TNF-R are also diminished rapidly. The latter observations confirm that both the proinflammatory and antiinflammatory arms of the cytokine cascade are TNFα dependent. This is discussed in detail elsewhere.[5,6] In rheumatoid synovial cultures in vitro the effect of anti TNFα antibody occurred subsequent to TNFα neutralization. In vivo, there is the interesting possibility that some of the cells producing TNFα which have TNFα on their surface prior to it being cleaved by "TNFα convertase," may be killed by the antibody in the presence of complement from serum or by antibody-dependent cell-mediated cytotoxicity. Such events can be shown in vitro.[7]

Recruitment of leucocytes to inflammatory sites is an essential step in permitting the inflammation to develop. This is a complex process, involving both adhesion molecules of various families (selectins, integrins, and so forth) as well as chemotactic factors, with chemokines being the most abundant. It was found that cA2 therapy diminished the expression of adhesion molecules E selectin, ICAM-1, and VCAM-1 in various assays, and that chemokine production such as IL-8 and MCP-1 was reduced, indicating downregulation of parameters for leucocyte recruitment.[8] Formal proof of diminished trafficking has come from studies with radiolabeled leucocytes (Taylor et al, in preparation).

Synovium in chronic rhematoid arthritis is very vascular, and to support the mass of synovium, angiogenesis is required. Anti-TNFα antibody therapy was found to downregulate the levels of vascular endothelial growth factor, suggesting that angiogenesis may also be regulated by the inflammatory response. Support for that concept was also found in vitro.[8]

The conclusion from these studies is that in a chronic immune-driven inflammatory response there are a number of pathways that become engaged and can serve to sustain the inflammatory process. Those delineated above are not necessarily the only ones.

IMPLICATIONS FOR OTHER DISEASES

Local recruitment of leucocytes to the disease site occurs in many diseases. It is regulated by the same families of adhesion molecules and chemokines as in rheumatoid

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New Immunosuppressants: Testing and Development in Animal Models and the Clinic: with Special Reference to DSG

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INTRODUCTION

Since the start of clinical transplantation in the late '50s and early '60s the development of immunosuppressive regimens has taken two main directions, the first of which has involved the search for new immunosuppressive drugs. Experiments, mainly in rodents and dogs, have been used to discover and test new immunosuppressive drugs. The drugs azathioprine, cyclosporine A, FK506, and RS61443 have all reached the clinic because of being effective as monotherapy in experimental animals. Secondly, in conjunction with their introduction into the clinic, the drugs mentioned above have mostly been used either in combination with steroids or in various combinations one with another. Their efficacy in monotherapy has been well established but the scientific support for using these drugs in combinations has sometimes been rather weak (Starzl et al. 1963, Klintmalm et al. 1981). This is easy to understand, as the requirements demanded of clinical studies aimed at developing new immunosuppressive drugs have increased, mainly because the results are far better than in the '60s and '70s. Thus, studies require many more patients per study group to test a new immunosuppressive drug today than they did 10 years ago when cyclosporine (CyA, European multicentre trial group 1982 and Canadian multicentre study group 1985) was introduced.

The first part of this presentation will discuss the possibilities for testing new immunosuppressive drugs in a rodent model, and further to test optimal drug

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Of Mice and Not Men: Differences between Mouse and Human Immunology

Javier Mestas and Christopher C. W. Hughes[1]

Mice are the experimental tool of choice for the majority of immunologists and the study of their immune responses has yielded tremendous insight into the workings of the human immune system. However, as 65 million years of evolution might suggest, there are significant differences. Here we outline known discrepancies in both innate and adaptive immunity, including: balance of leukocyte subsets, defensins, Toll receptors, inducible NO synthase, the NK inhibitory receptor families Ly49 and KIR, FcR, Ig subsets, the B cell (BLNK, Btk, and λ5) and T cell (ZAP70 and common γ-chain) signaling pathway components, Thy-1, γδ T cells, cytokines and cytokine receptors, Th1/Th2 differentiation, costimulatory molecule expression and function, Ag-presenting function of endothelial cells, and chemokine and chemokine receptor expression. We also provide examples, such as multiple sclerosis and delayed-type hypersensitivity, where complex multicomponent processes differ. Such differences should be taken into account when using mice as preclinical models of human disease. *The Journal of Immunology*, 2004, 172: 2731–2738.

Mice are the mainstay of in vivo immunological experimentation and in many respects they mirror human biology remarkably well. This conservation of function is reflected in recent reports on the sequencing of both the human and mice genomes, which reveal that to date only 300 or so genes appear to be unique to one species or the other (1). Despite this conservation there exist significant differences between mice and humans in immune system development, activation, and response to challenge, in both the innate and adaptive arms. Such differences should not be surprising as the two species diverged somewhere between 65 and 75 million years ago, differ hugely in both size and lifespan, and have evolved in quite different ecological niches where widely different pathogenic challenges need to be met—after all, most of us do not live with our heads a half-inch off the ground. However, because there are so many parallels there has been a tendency to ignore differences and in many cases, perhaps, make the assumption that what is true in mice—in vivo veritas—is necessarily true in humans. By making such assumptions we run the risk of overlooking aspects of human immunology that do not occur, or cannot be modeled, in mice. Included in this subset will be differences that may preclude a successful preclinical trial in mice becoming a successful clinical trial in human.

In this review our aim is not to suggest that the mouse is an invalid model system for human biology. Clearly, with so many paradigms that translate well between the species, and with the relative ease with which mice can now be genetically manipulated, mouse models will continue to provide important information for many years to come. Rather, our aim is to sound a word of caution. As therapies for human diseases become ever more sophisticated and specifically targeted, it becomes increasingly important to understand the potential limitations of extrapolating data from mice to humans. The literature is littered with examples of therapies that work well in mice but fail to provide similar efficacy in humans (2–7). By focusing on some known differences between mouse and human immunology we hope to spur interest in this area and encourage others to note differences where they occur.

Structure and general characteristics

The overall structure of the immune system in mice and humans is quite similar. As this topic has been recently reviewed in depth (8), we will not go into great detail here. One difference worth noting is that whereas mice have significant bronchus-associated lymphoid tissue, this is largely absent in healthy humans (9), possibly reflecting a higher breathable Ag load for animals living so much closer to the ground.

The balance of lymphocytes and neutrophils in adult animals is quite different: human blood is neutrophil rich (50–70% neutrophils, 30–50% lymphocytes) whereas mouse blood has a strong preponderance of lymphocytes (75–90% lymphocytes, 10–25% neutrophils) (10). It is not clear what, if any, functional consequence this shift toward neutrophil-rich blood in humans has had.

Tyrosine kinase receptor expression on putative hemopoietic stem cells (HSC)[2] shows a reciprocal pattern, with mouse HSC being predominantly c-*kit*^{high}, *flt-3*$^{-}$, whereas human HSC are predominantly c-*kit*^{low}, *flt-3*$^{+}$ (11).

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[2] Abbreviations used in this paper: HSC, hemopoietic stem cells; iNOS, inducible NO synthase; γ_c, common γ-chain; DETC, dendritic epidermal T cells; MS, multiple sclerosis; DTH, delayed-type hypersensitivity; EC, endothelial cells.

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Opinion

Satisfaction (not) guaranteed: re-evaluating the use of animal models of type 1 diabetes

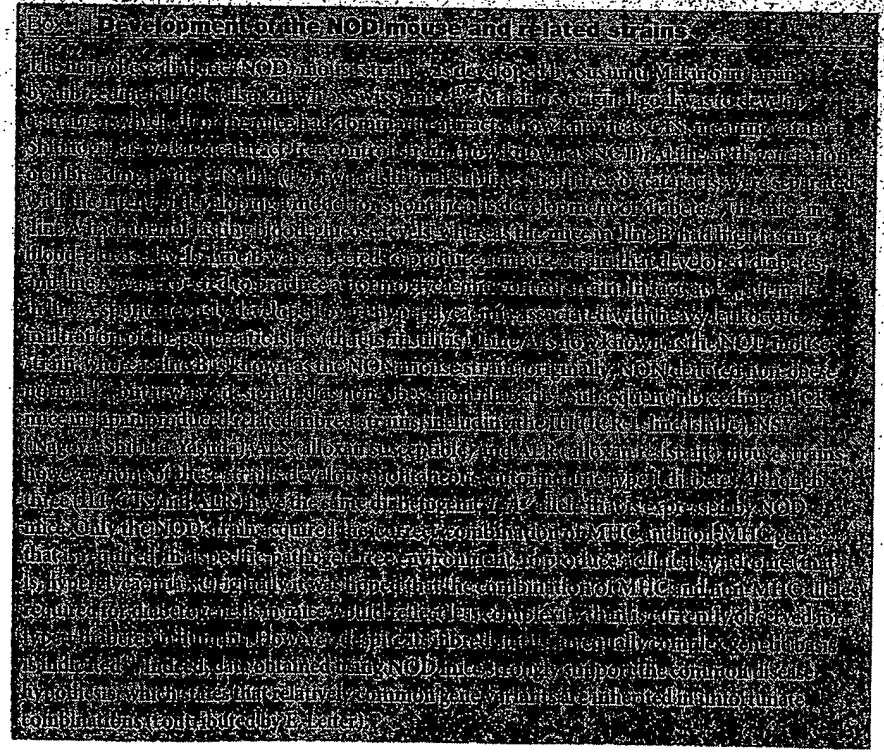
Bart O. Roep, Mark Atkinson and Matthias von Herrath

Abstract | Without a doubt, rodent models have been instrumental in describing pathways that lead to pancreatic β-cell destruction, evaluating potential causes of type 1 diabetes and providing proof-of-principle for the potential of immune-based interventions. However, despite more than two decades of productive research, we are still yet to define an initiating autoantigen for the human disease, to determine the precise mechanisms of β-cell destruction in humans and to design interventions that prevent or cure type 1 diabetes. In this Perspective article, we propose that a major philosophical change would benefit this field, a proposition that is based on evaluation of situations in which rodent models have provided useful guidance and in which they have led to disappointments.

Human type 1 diabetes (T1D; formally known as insulin-dependent diabetes mellitus) is thought to be an autoimmune disease in which, in genetically predisposed individuals, autoaggressive $CD4^+$ and $CD8^+$ T cells invade the pancreatic islets of Langerhans and destroy the insulin-producing β-cells[1]. The resulting lack of control of glucose levels in the blood results in hyperglycaemia, which leads to severe chronic complications with time: most notably, widespread vascular damage that results in kidney failure, blindness, heart disease and chronic ulcers. A prevention or cure for this disease has been sought for more than a century. However, even now, treatment for T1D only involves insulin substitution, and although this is life-saving, it can only delay and not prevent disease-associated complications in most patients.

Studying the pathogenesis of T1D directly in humans is difficult because of several factors: the asymptomatic course of the disease process before clinical manifestation; the remote, retroperitoneal location of the pancreas; and the fact that immunological events in the islets are neither easily detectable nor reflected in the peripheral blood. Therefore, in the mid- to late 1970s, investigators sought to develop improved and more-accurate immunological models for T1D. Until then, no animal model had been developed that showed a reproducibly high incidence of diabetes and allowed for study of the disease in a timely experimental manner. Great enthusiasm arose when, about a decade before the development of transgenic and engineered animal models of T1D, the non-obese diabetic (NOD) mouse was discovered (BOX 1). Such enthusiasm was certainly justified. NOD mice develop spontaneous diabetes that seems to reflect many crucial aspects of the human disease, including development of islet-specific autoantibodies and inflammation of the pancreatic islets[2]. All spontaneously

Box 1 | Development of the NOD mouse and related strains



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Box 1 | Development of the NOD mouse and related strains

The non-obese diabetic (NOD) mouse strain was developed by Susumu Makino in Japan by inbreeding JcICR (also known as Swiss) mice[3]. Makino's original goal was to develop a strain in which all of the mice had dominant cataracts (now known as CTS, meaning cataract Shionogi), as well as a cataract-free control strain (now known as NCT). At the sixth generation of inbreeding the CTS line (F6), two additional sublines, both free of cataracts, were separated with the intent of developing a model for spontaneous development of diabetes. The mice in line A had normal fasting blood-glucose levels, whereas the mice in line B had high fasting blood-glucose levels. Line B was expected to produce a mouse strain that developed diabetes, and line A was expected to produce a normoglycaemic control strain. In fact, it was female mice in line A spontaneously developed overt hyperglycaemia associated with heavy leukocytic infiltration of the pancreatic islets (that is insulitis). Line A is now known as the NOD mouse strain, whereas line B is known as the NON mouse strain (originally NON denoted non-obese normal). In 1974, redesignated as non-obese non-diabetic. Subsequent inbreeding of ICR mice in Japan produced genetically inbred strains, including the JcI:(ICR) (line 1 subline) NSY (Nagoya-Shionogi-Yasuda), ALS (alloxan susceptible) and ALR (alloxan resistant) mouse strains. However, none of these strains develops spontaneous autoimmune type 1 diabetes, although three (JcI:ICR, CTS and ALR) have the same diabetogenic *I-Ag7* allele that is expressed by NOD mice. Only the NOD strain acquired the correct combination of MHC and non-MHC genes that is required, in a specific-pathogen-free environment, to produce a clinical syndrome (that is, hyperglycaemia). Originally it was hoped that the combination of MHC and non-MHC alleles required for diabetogenesis in mice would reflect (be as complex) that is currently observed for type 1 diabetes in humans. However, despite its inbred status, an equally complex genetic basis is indicated[4]. Indeed, data obtained using NOD mice strongly support the common disease hypothesis, which states that relatively common gene variants are inherited in unfortunate combinations (contributed by F. Leiter).

diabetic animal models have a crucial dependence on MHC alleles; indeed, one of the main disease-susceptibility loci in the NOD-mouse model, the *I-A*^{g7} allele, is an orthologue of the high-risk-conferring *HLA-DQ0302* allele in humans. As a result, it was assumed that T1D was probably a CD4$^+$ T-cell-mediated autoimmune disorder, in which the MHC class II susceptibility alleles would be crucial in presenting autoantigens and in determining the selection of autoaggressive specificities[3]. Further support for this idea was provided by the isolation from NOD mice of CD4$^+$ T-cell clones that induced diabetes when transferred to immunodeficient recipients[4–6], so an immunologically based cure for T1D seemed to be within reach. Various aspects of the disease were also investigated using a large range of genetically engineered rodent models of the disease. However, researchers soon realized that, unfortunately, T1D was much more complex than expected: research in the 1990s revealed that there are many non-MHC-linked disease-susceptibility genes[7]. It was also difficult to define the specificity of the T cells that initiate disease[8] and to identify distinct environmental causes[9]. In addition, many of the preventive therapies that seemed to be promising in NOD mice did not show similar efficacy in human patients[2]. Given these developments, we propose that a major shift in thinking would be beneficial for research into T1D. We believe that it is necessary to re-evaluate and consider at a higher level the apparent differences in the immunology and biology of T1D between mice and men, as well as to more carefully consider the potential experimental pitfalls in both spontaneous and engineered animal models of disease. Furthermore, we believe that this re-evaluation will allow crucial, additional lessons to be learned from animal models of T1D that (hopefully) will delineate a road map to a cure for the disease.

Table 1 | **Comparison of autoimmune diabetes in NOD mice and humans**

	Humans	Mice
Similarities		
Genetic predisposition and polygenetic trait	Yes	Yes
MHC loci contribution	Multiple	Multiple
Environmental influence	Yes	Yes
Defective peripheral immune regulation	Yes	Yes
Impaired dendritic-cell maturation and function	Possibly	Yes
Disease transmission with bone-marrow transplantation	Yes	Yes
Autoantigens	GAD65, IA2, insulin and 38 kD	GAD65, IA2, insulin and 38 kD
Initiating autoantigen	Unknown	Unknown
Delayed onset with immunosuppression	Yes	Yes
Protection mainly linked to early gluten exposure	Yes	Yes
Differences		
Endogenous retrovirus in β cells	Unclear	Yes
T-cell-driven insulitis	Mild	Severe
Human reactivity to β cells	GAD65, IA2 and insulin	Insulin
Insulin gene	One	Two
GAD65 expression by β cells	Yes	No
Incidence	0.25–0.40%	> 80%
Incidence in genetically susceptible subjects	5–30%	> 80%
Gender bias	No	Females
Peri-insulitis	No	Yes
Lymphocytic infiltrates in other tissues	Minority of individuals	All mice
Susceptibility of β cells to STZ or NO in vitro	Only at high concentrations	Very susceptible
Maternal autoantibodies	Potentially reduced risk of T1D	Diabetogenic
B cells required	No	Yes
Successful intervention therapies	Pending	> 195

*Reference 114. ‡Reference 115. GAD65, glutamic-acid decarboxylase 65; IA2, islet-cell antigen 2; NO, nitric oxide; NOD, non-obese diabetic; STZ, streptozocin.

Rodent models of spontaneous T1D

Since their initial description more than two decades ago, spontaneous animal models of T1D — namely, the biobreeding (BB) rat and the NOD mouse — have been surrogates for investigating the immune responses that are associated with the human disorder. Such animal strains are unique models of diabetes that are equivalent to assessing multiple copies of a single individual, so they cannot be compared directly to the heterogeneous human population. The disease in NOD mice has many similarities with the human disorder; however, there are also important discrepancies[1] (TABLE 1). One such difference relates to the phenotype of insulitis. In female NOD mice, the lymphocytic infiltrate is extensive and is indicative of an ectopic lymph node developing around the pancreatic vasculature[1]. By contrast, in most cases, human insulitis seems to be more 'delicate', with few leukocytes detectable in the inflamed islets[2]. Indeed, the human lesion is more accurately modelled by BB rats or transgenically engineered animal models of T1D, which show a focused islet inflammation that appears shortly before clinical disease[2].

Another divergence between animal models and humans is that autoantibodies that are specific for islet antigens other than insulin are almost completely absent in NOD mice, if one restricts consideration to those antigens validated in blinded workshops — that is, insulin, glutamic-acid decarboxylase (GAD) and islet-cell antigen 2 (IA2)[11]. By contrast, autoantibodies that are specific for GAD or IA2, and particularly the presence of both of these, are strong predictors of onset of T1D in humans[12]. Furthermore, a recent report identified the presence of maternal islet autoantibodies as a diabetogenic factor in the offspring of diabetic mice[13,14]. In humans, the situation seems to be the inverse: transplacental transfer of autoantibodies has frequently been observed, but this correlates with protection from islet-specific autoimmunity[15]. Despite these differences, further studies of NOD mice might provide us with important clues for understanding the pathogenic roles of autoantibodies in humans, which are unknown at present. (For example, these autoantibodies could potentially capture β-cell proteins and thereby increase the presentation of β-cell-derived peptides by B cells.)

Both the NOD mouse and the BB rat have innate immune-system defects that have been well characterized, including defects in the

maturation and function of natural killer cells, macrophages and dendritic cells[3]. Such defects are not uniformly present in humans with T1D[16]. On the basis of these immune defects, the development of T1D, in the NOD mouse in particular, seems to be a 'default' pathway, disrupted only in the presence of encounters with the microbial environment[17]. Consistent with this, NOD mice need to be kept under specific-pathogen-free conditions to develop the predictable high frequency of diabetes. This feature might have an important message for our understanding of T1D development in certain individuals, in whom a lack of early childhood infections might contribute to the general risk of developing autoimmune diseases and allergic asthma, but this is certainly not the case for all patients[18–20]. Conversely, viral infections have been shown to trigger diabetes in diabetes-resistant strains of BB rats and in transgenic animal models[21–24], and they are thought to induce diabetes in at least some patients. Therefore, to gain insight into the role of viruses in the development of T1D, studies of NOD mice should be complemented by studies using other models. Additional marked differences between the immune systems of rodents and humans have been comprehensively summarized[25] and include important discrepancies in both innate and adaptive immunity. From a list of more than 80 differences, more-specific examples include variations in the balance of leukocyte subsets, antigen-presenting-cell defects and dysregulation of thymic selection[25]. It is easy to envisage that this large number of disparities between species would have a considerable impact on immune processes, including those leading to autoimmune disorders such as T1D.

Despite this extensive list of immunological differences and the inbred status of animal models compared with the genetically heterogeneous at-risk human population, several pathways that can mediate β-cell destruction (both important pathways and redundant pathways) have been defined[26–43]. These include roles for $CD4^+$ and $CD8^+$ T cells, natural killer T cells, co-stimulation, presentation of islet autoantigens, and pro- and anti-inflammatory cytokines in β-cell destruction and thereby the development of autoimmunity in animals models of T1D. However, the mechanisms of β-cell destruction in humans remain elusive, and it is possible that, in humans, there are differences in the relative importance of individual mechanisms for disease initiation and progression. This lack of knowledge about human T1D is not a shortcoming of animal models but is associated with our lack of understanding about the subclinical disease process in humans and the inaccessibility of the pancreatic lesions in humans. Indeed, immune mechanisms that are identified in NOD mice to cause islet destruction almost certainly include those mechanisms that are responsible in humans, mainly because nearly all immune mechanisms tested have been identified as having a role in NOD mice, perhaps with the exception of direct lysis by complement. Whether heterogeneity in pathophysiological pathways occurs because of the multiplicity of genotypes present in humans with T1D remains to be analysed. However, elucidating the underlying mechanisms of spontaneous development of T1D in each unique animal model, whether spontaneous or engineered, will continue to allow clinical investigators insight into the spectrum of potential pathways that lead to T1D in humans.

Clearly, there are many important lessons still to be learned from animal models of T1D. Unfortunately, the interpretation and extrapolation of relevant data from animal models to clinical practice, in terms of disease prevention, have often been incorrect or proven unsuccessful. But why is this the case? As mentioned previously, NOD mice and other animal models of T1D might reflect the events of only a subset of the human population with T1D. The variation in clinical manifestations might therefore require more individualized and customized treatments. Furthermore, the possibilities for prevention that have been discovered using NOD mice have not all been tested in humans. It seems reasonable to speculate that the same positive results observed in NOD mice would occur in humans if these treatments were applied. An important question is whether such treatments are acceptable in therapeutic settings, in terms of both the risk and the ethics. In addition, although translation of prevention-based therapies from animal models to humans has been difficult, application of methods for disease reversal might prove more favourable with time, because preliminary results from studies of a few agents (discussed later), when tested in NOD mice with recent onset of diabetes[44–48], have shown effectiveness in pilot studies of humans[49,50]. Clearly, our lack of knowledge regarding the roles of effector T cells, regulatory T cells and islet-resident antigen-presenting cells in human T1D is a shortcoming of both studies of prevention and disease reversal. Indeed, more emphasis should be placed on studies of the immune system in humans and, in particular, the mechanisms of T-cell regulation and dysregulation that arise in health and disease. We have little knowledge of how to achieve the desired activation and migration of regulatory T cells at the appropriate time to sites of inflammation in humans; however, such studies are a strength of animal models. Animal models should also lead to improving the imaging of insulitis, developing diagnostics and testing stem-cell technologies for β-cell-replacement therapy. The application of such studies in larger animals will also be required; therefore, a search for new models of spontaneous T1D in larger animals is warranted.

Caveats of engineered animal models

Genetic manipulation of rodents and the generation of gene-'knockout' animals continue to highlight the multiplicity of pathways that, when perturbed, can produce diabetes or resistance to diabetes. Disease models using such transgenically engineered and gene-knockout animals can be important tools if they are used correctly, even though, inherently, they are not the equivalent of the naturally occurring disease[51–57] (TABLE 2). For example, transgenic animal models have been useful for evaluating the potential redundancies and relative efficacy of the multiple pathways that can lead to β-cell death[22,26,68,69]. In addition, they have enabled researchers to determine the precise numerical balance of autoaggressive effector cells versus regulatory cells and to understand that disturbance of this balance can lead to T1D[62,70–73]. Furthermore, we now know that a high number of antigen-specific T cells is required to drive the autoantigen-specific response in hosts who are not genetically predisposed to disease, particularly if only one or a small number of β-cell autoantigens are targeted[72–75]. By contrast, treatment with relatively small numbers of regulatory cells can be effective in preventing disease[62,71].

Several important issues, however, must be considered when utilizing such models. First, the transgenic overexpression of cytokines and chemokines by β-cells frequently results in variable findings, which depend on the precise time of expression with respect to the ongoing autoimmune response that precedes the clinical onset of hyperglycaemia and diabetes. For example, tumour-necrosis factor can have opposite effects when expressed at the start of an autoimmune attack (enhancement of disease[76,77]) and when expressed later, when most β-cells have been destroyed and islets are fully infiltrated (reduction of disease[33]). So, it is necessary to be more cautious when considering the direct use of such cytokines for human therapy and to rationally and safely correlate the different stages in

rodent pre-diabetes and in human patients who are at risk of developing disease (that is, genetically prone individuals with antibodies specific for one or more islet antigens) — a task that has not yet been fully accomplished.

Second, mice that are genetically deficient in these same cytokines or chemokines do not always have the expected phenotype opposite of the transgenic animal[78–82]. One reason for this difference is that living organisms can frequently compensate for a genetic defect; this might even be the case for animals with conditional gene knockouts that take effect during adult life. Alternatively, it might be that the local or systemic level of the cytokine in relation to the disease process might vary for each cytokine or chemokine. Therefore, cytokines that are expressed at low baseline levels might not affect the natural disease process leading to T1D (as observed for interleukin-4 (IL-4)-deficient mice, which are as susceptible to diabetes as wild-type animals), but cytokines might be protective if overexpressed or produced by regulatory T cells (as occurs for NOD mice that are transgenic for *Il-4* expressed under the control of the rat insulin promoter, which are less susceptible to disease than control NOD animals[83], and as occurs after retroviral transfection of NOD mouse T cells with *Il-4* cDNA[84]). In addition, the systemic level of certain cytokines might diverge between some experimental mouse models and patients with T1D. For example,

Table 2 | **Examples of engineered models of autoimmune diabetes**

Gene type/function	Gene(s)	Outcome	Clinical equivalent/relevance	Refs
Transgenic mice				
TCR	BDC2.5×NOD 8.3×NOD gp33-specific TCR HA-specific TCR OT-1 TCR	In some cases, acceleration of T1D occurs. Spontaneous diabetes occurs in non-NOD mice, except in dual TCR transgenic mice expressing HA under the RIP. Peripheral non-destructive insulitis occurs.	Presence of T cells with specificity for islet antigens does not always lead to breaking of tolerance. Background has profound influence on disease. Non-destructive autoimmune insulitis does not commonly occur in humans.	54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77
MHC	HLA-DR HLA-DQ HLA-A2 I-E^{d} I-A^{g7Asp*}	No spontaneous T1D when overexpressed by β-cells, unless β-cells are overloaded with neo-proteins. Humanized mice can be important models.	MHC class II contributes to T1D but does not lead to breaking of tolerance. Suitable for studying human MHC-class II-restricted T cells and MHC-associated genetic protection.	69, 116, 117
Neoantigen	HA HEL gp33 OVA	Non-tolerant antigens alone do not lead to autoimmunity. T cells need to be activated in sufficient numbers to lead to significant β-cell destruction.	Neoantigens alone do not lead to autoimmunity, inflammation is required. In RIP–LCMV transgenic mice, viral infection constitutes a sufficiently strong stimulus for T1D development. Similarly, cross-presentation can be enough for propagating the autoimmune process.	54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77
Co-stimulation	*Cd80* *Cd86* *B7h1*	In some cases, spontaneous diabetes can occur when these molecules are expressed under the RIP. T1D development is markedly accelerated.	Local clonal expansion of lymphocytes in islets promotes T1D. If β-cells function as professional APCs, T1D development is accelerated.	118, 119
Cytokine	*Ifn-α* *Ifn-γ* *Tnf* *Tnf* and *Cd80*	All of these accelerate T1D development. IFN-γ or TNF plus CD80 overexpression by β-cells can lead to spontaneous diabetes without it other stimuli, together with presentation of autoantigens.	Inflammatory cytokines such as these are expected to accelerate development of human T1D. Contraction of virus could be explained by this.	56, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 80, 81
Anti-apoptosis	*Fas* *Bcl-2*	No marked prevention of T1D in most models.	Redundant mechanisms of β-cell destruction exist.	26, 75, 120, 121
Autoantigen	*Gad65*^{T} *Gad67*^{T} Insulin	GAD65 expression in the thymus and the ensuing tolerance does not prevent T1D. Insulin expression in the thymus causes strong reduction of T1D incidence.	There is more than one autoantigen that can drive the autoimmune process, showing the importance of redundancy. Insulin is a strong primary candidate autoantigen.	54, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 110
Knockout mice				
Autoantigen	*Gad65*§ Insulin 1 Insulin 2	GAD-anti-sense studies (which prevent diabetes) are not supported by follow-up studies inducing thymic tolerance to GAD. Differential effect on T1D depending on which autoantigen is deleted (accelerated in insulin 1 knockout, promoted in insulin 2 knockout).	Different autoantigens have different roles in the autoimmune process. Similar to many of the known autoantigens, GAD might be non-essential for T1D. Mouse β-cells do not express GAD65, human β-cells do, humans do not have the gene encoding insulin 2.	56, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77
Cytokine	*Il-4* *Il-10* *Tgf-β* *Ifn-γ*	Acceleration of T1D in most, but not all, cases.	Regulatory cytokines that dampen T1D exist and can be used therapeutically.	58, 78, 79, 122, 123

*Mutation of residue 57 of the MHC I-A^{g7} β-chain to aspartic acid leads to a mutant MHC molecule that protects against diabetes. ‡Expressed only in the thymus. §Using antisense GAD transgene not gene deletion. APCs, antigen-presenting cells; *Bcl-2*, B-cell lymphoma 2; *Gad*, glutamic-acid decarboxylase; gp33, a peptide corresponding to amino-acid residues 33–41 from the LCMV glycoprotein; HA, haemagglutinin; HEL, hen-egg lysozyme; *Ifn*, interferon; *Il*, interleukin; LCMV, lymphocytic choriomeningitis virus; NOD, non-obese diabetic; OVA, ovalbumin; OT-1 TCR, TCR specific for an OVA peptide in the context of MHC class I; RIP, rat insulin promoter; T1D, type 1 diabetes; TCR, T-cell receptor; *Tgf-β*, transforming growth factor-β; *Tnf*, tumour-necrosis factor.

IL-2-deficient NOD mice develop widespread autoimmunity because IL-2 production is required for T-cell apoptosis and regulatory T-cell development[85], and a priori, T cells from NOD mice produce less IL-2 on a per cell basis[86]. This is consistent with the suppression of diabetogenesis that is achieved by long-term treatment of NOD mice with recombinant IL-2 (REF. 86). However, it seems that a low level of IL-2 production on an individual T-cell basis is not a direct marker for pre-diabetes in humans[87]. By contrast, overexpression of IL-2 promotes diabetes in transgenic models[88]. In better agreement with this finding, administration of antibody specific for the IL-2 receptor has shown promise in islet transplantation[89] and is currently under evaluation in humans with T1D, using a combination therapy with mycophenolate mofetil (see Type 1 Diabetes TrialNet in the Online links box). So, experimentally deleting a given factor cannot be expected to result in the opposite outcome of increasing it locally and/or systemically. Therefore, extrapolating data obtained from rodent models to the use of cytokines or chemokines in human interventions (both local and systemic) will require precise and detailed knowledge of the cellular circuits and functions that are crucial for therapeutic success so that the same effect can be sought and tracked during a clinical trial. It will also be important that the mechanisms described for transgenic mice are validated in at least one other animal model: for example, NOD mice.

Third, it is of crucial importance when using engineered animal models of T1D to consider that the way in which the disease manifests depends on the genetic background of the mouse. Therefore, backcrossing studies need to be well controlled, using carefully selected and appropriate littermate controls and sufficiently high numbers of animals. It is known that 'background' genes can still influence disease penetrance after more than ten backcrosses[40,90]. Fourth, a general caveat is that overexpression of immune mediators in β-cells can create situations in which the expression of transgenes driven by the rat insulin promoter might be at such a high level, using most of the transcriptional machinery, that it prevents endogenous gene expression by β-cells, thereby directly increasing the vulnerability of the animal or even affecting its survival. This can be circumvented by using appropriate controls for insulin secretion and *in vitro* assays that validate normal β-cell function.

As a result of studies using both spontaneous and engineered animal models of T1D, researchers now have a better understanding and appreciation of why the complexity of T1D in both humans and animal models has been underestimated. There is no doubt that animal models have helped us to understand a multitude of pathophysiological mechanisms (BOX 2) and have greatly furthered our understanding of the many potential pathways that can lead to T1D. However, increasing complexities have emerged in the areas of genetics, immunology, immunotherapy and environmental factors, which can all modulate disease onset, and these have complicated the application of animal models to studies of human T1D.

The therapeutic dilemma

Given the increasing number of successes in preventing diabetes in NOD mice (BOX 2), an idea has grown among many in the T1D research community that everything prevents T1D in NOD mice. This idea, together with a limited number of high-profile failures of studies attempting to effectively translate therapeutic studies of T1D prevention from NOD mice to humans (for example, daily subcutaneous insulin injections in the Diabetes Prevention Trial[91] and nicotinamide in the European Nicotinamide Diabetes Intervention Trial[92]) have led some to question the relevance of animal models to studies of disease prevention. While appreciating why, amid this back-drop, the relevance of animal models is being questioned, we think that the overall concept is unfair, and we contend that important lessons on disease prevention can be learned from animal models, but only if the studies are subject to proper design and interpretation. When seeking to translate observations from animal models of disease to human therapies, several issues should be taken into account.

Exercise patience. The first study of a relevant animal model in which established disease was reversed was published in 1979 (REF. 93); in this study, lymphocyte-specific serum was used to reverse disease in BB rats. However, this approach has only recently been used

BOX 2 | Lessons from rodent models

Genetics

Rodent models of type 1 diabetes (T1D) have vastly improved our understanding of the genetic basis of T1D and have taught us to expect a comparable complexity for the genetics of T1D in humans. Although some of the immunopathophysiological phenotypes of various well established congenic rodent models are (mainly) similar, they have different sets of non-MHC susceptibility genes. It is thus now necessary to determine which of these genes have a role in the pathogenesis of human T1D.

Immunology

Despite the limitations of comparing the immune systems of different species, information gleaned from rodent models has proven useful. However, it remains to be confirmed whether the mechanisms of β-cell destruction that are identified in animal models of T1D mediate the destruction of β-cells in humans.

Initiating autoantigens

Although animal models have so far proved to be of limited use in determining the initiating autoantigens, these models have indicated that there might be a multiplicity of antigens, many of which were first identified in humans. The contribution of candidate autoantigens to the disease process has been elegantly evaluated using animal models. When humanized non-obese diabetic (NOD) mice, which are in development, become available, these animals could be used to examine the responses of HLA-matched human T cells. In contrast to several other animal models of human autoimmunity, it has proven difficult to induce or accelerate T1D in animals by immunization with a candidate autoantigen. This might convey the important message that strong as well as persistent antigenic stimuli are required, such as those provided by viral infections, dendritic cells and cross-presentation of islet antigens[94–97].

Immunotherapy

Approximately 15 published methods prevent or delay the development of T1D in NOD mice[98]. However, some of these interventions reflect mistakes in using the model, and others result from questionable study designs or tests in a single animal model (usually the NOD mouse), without validation of the observation in other suitable animal models. Full penetrance of the underlying genetic susceptibility of the NOD mouse was often not achieved. Furthermore, some interventions claimed to prevent diabetes but only retarded the age of onset, and other studies were terminated at only 20–30 weeks of age. Nonetheless, there are several seminal examples of therapies that have been derived from animal models and have clinical efficacy or promise[98].

successfully in humans[94]. Another seminal example of the proper use of animal models to intervene in clinical autoimmune disease is the blockade of tumour-necrosis factor in patients with rheumatoid arthritis[95].

Exercise caution and apply stringency tests. It is clear that NOD mice are at the 'razor's edge' in terms of disease development because they have a fine immunological balance, with a disease pathology that is easily tipped towards prevention if therapeutic interventions or environmental perturbations are begun early in life[2]. Unfortunately, early intervention at 4–6 weeks of age is common to most studies of disease prevention carried out using NOD mice. As a result, most of the immunological 'sledge-hammers' that show therapeutic efficacy in NOD mice (and lead to the idea that 'everything prevents') will never be applied to humans because the developmental equivalents of 4–6-week-old mice are human infants and young adolescents. Fortunately, corrective action to rectify this situation by the proper use of animal models should be easy for the T1D research community to undertake (TABLE 3). Specifically, future studies using animal models should use agents that are appropriate for application in humans at the particular stage of disease development that is, more-benign agents (for example, vitamin supplements) early in the pre-symptomatic phase of the disease, and agents that are more toxic (for example, immunosuppressive agents) at, or immediately before, disease onset. In addition to this matching of ethics and safety, the financial costs of such interventions should also be included and should show a degree of feasibility. Only a minority of published studies of animal models of T1D have investigated the safety, dosage and toxicity of the treatment administered. Clearly, as well as studying relevance to human disease, more attention should be given to the effects of an agent on the immune response, physiology, metabolism and overall health of the animal.

Validate in more than one model: n > 1. Because few patients with T1D show the same clinical features as NOD mice, studies in other animal models of the disease are required. It is notable that therapies for diabetes that have been identified in NOD mice using GAD or GAD-derived peptides are rarely tested in rat models of diabetes, even though rat islets are more human-like in terms of the levels of GAD protein expressed by β-cells[96]. Similarly, favourable results that show the suppression of diabetes in NOD mice before disease onset following the injection of peptide 277 from heat-shock protein 60 (HSP60) or following the feeding of nicotinamide have apparently not been followed up with trials in rats or in other animal models of diabetes[97]. Similar issues apply to the use of NOD mice to validate therapies tested in transgenic models. Even when data from several mouse and rat models support the extrapolation of a therapy to humans (for example, prophylactic treatment with insulin[98,99]), extreme caution must still be exercised (for example, in relative terms, more insulin was required for retardation of diabetes in NOD mice than can be safely administered to pre-diabetic individuals).

Consider treatment of recent-onset T1D as a goal. It must also be emphasized that, in contrast to the extensive number of therapies that reportedly prevent disease in NOD mice, only a few therapies (for example, antibodies specific for CD3 or CD4, lymphocyte-specific globulin, complete Freund's adjuvant (CFA) plus C57BL/6 splenocytes, or HSP60-derived peptide) have shown efficacy when administered either late in the disease process (that is, 12 weeks of age or later) or at the time of symptomatic onset of disease[47,48]. Furthermore, only a fraction of the interventions tested in NOD mice are efficacious in other rodent models that have a more rapid onset of disease[2]. So, these more-stringent experimental situations might be better suited for defining interventions that have potential for treating human T1D. We think that, rather than being a negative feature, this might provide evidence for the relevance of rodent models to human T1D, because in general, T1D seems more difficult to reverse in humans than in rodents. In addition, this difficulty is even more marked after hyperglycaemia has been established. However, we think that there is a practical benefit to investigations, because at present, most studies are aimed at disease reversal, and many of these studies form part of organized efforts to avert disease (see Type 1 Diabetes TrialNet and Immune Tolerance Network in the Online links box). That is, NOD mice and other rodent models can provide a degree of similarity that should be a feature to take advantage of in studies that aim to prevent disease.

In the end, a more-balanced approach to rodent models could be based on the assertion that each model is only representative of a single case report. Ironically, there are interesting clinical counterparts of gene-knockout models, because there are patients with genetic deficiencies that provide specific information on certain pathways of a

Table 3 | **Roadmap to improved use of animal models for studying human T1D**

Observation	Appropriate action
Early prevention of disease (for example, at 4 weeks of age) in NOD mice is easy	Focus attempts at early intervention on agents that are suitable for such use in humans (with respect to ethics, safety and cost)
Non-specific pathogens influence the disease rate in NOD mice	Carry out investigations in specific pathogen-free environments
Late interventions for disease prevention or reversal in NOD mice are difficult	Attempt more studies of agents at the onset of disease, with the goal of disease reversal or retention of C-peptide function
Most disease interventions are safe in NOD mice; they can induce sickness in humans	Test dosage and toxicity
Many reports use the word 'prevent' but 'delay' might be more appropriate	Establish criteria that define 'marginal delay', 'significant delay' and 'absolute prevention'
Most studies of type 1 diabetes in animal models use NOD mice, a practice that carries risks for application to type 1 diabetes in humans	Attempt prevention-based interventions using other animal models (that is, rats) and/or other mouse models
Animal models are not chosen according to the questions asked but based on their frequency of use in the scientific community	Re-evaluate the relevance of each model to the questions asked. Avoid making animal models 'gold standards' for all questions asked
Animal models are used to replicate genetic defects (using gene knockouts) in the immunopathogenesis of human type 1 diabetes, although humans do not necessarily show the defect(s) that causes diabetes in the animal model	Avoid this type of chicken or egg loop and validate immune defects first in humans; then replicate the finding made in the animal model while citing the appropriate caveats for translating this data to apply to humans. Gene-knockout models will be useful tools to dissect immunological pathways, yet few human diseases are based on a single defect

NOD, non-obese diabetic.

disease[100]. However, such clinical case reports are often met with the scepticism that 'those patients are the exception' and that population studies should be carried out to determine the clinical relevance'. Given the concerns with spontaneous disease models, these clinical reports should have an equal position. The presumption of researchers that animals with spontaneously occurring disease more closely resemble (and therefore are better models of) complex diseases in humans (for example, T1D) than models of induced diseases might not always be correct.

The need for a change in attitude

We think that there is an immediate need for a cultural change when carrying out research that will be translated from mice to men and that this will, in certain cases, need to be associated with differences in funding structures. With this idea, we do not want to imply that mistakes were made in the past; instead, we want to put forward the idea that translational studies have arrived at a stage at which further changes will be beneficial. The scientific community needs to find ways to reward individuals who are involved in larger team efforts and to provide career pathways that are not based solely on accumulating publications with high impact. Peer-reviewed funding mechanisms that support such efforts are already on the rise[101]. We now detail the reasons for this philosophical and structural change in how data obtained from studies of animal models are used.

Given the ease of successful retardation of diabetes by intervention at an early stage of immune attack on the islets (a stage that precedes overt clinical hyperglycaemia in the NOD mouse), it is difficult to evaluate claims that a reagent has potential for preventing diabetes in humans if there is no accompanying evidence that it is effective in one of the rat or transgenic mouse models. Overall, making one animal model the 'gold standard' and discounting findings in alternative models that might better address certain questions or show the 'universality' of a prophylactic intervention or therapeutic treatment should be avoided. This change would require funding agencies to allow investigators to have maintenance costs for colonies of rodents used in several distinct models, or it would force investigators studying different models to collaborate in running trials.

Finally, it is essential to validate important findings in humans. This requires more resources and needs larger groups to work in cooperation. Therefore, the funding mechanisms, as well as the scientific culture in the field of biology, needs to change drastically. Individual careers should not be evaluated on the basis of publications and their impact but on contributions to a team and the ability of an individual to cooperate, contribute and work in larger teams. Authorship rankings should take second place. This will be essential to avoid animal models developing dynamics of their own and functioning as sole trend-setters in immunology, which so far has not resulted in a cure for T1D.

Relevance to autoimmunity models

These experiences are certainly not unique to research using animal models of T1D. Yet research into T1D is somewhat privileged by having access to several models of spontaneous autoimmune disease and by being able to assess autoimmunity to peripheral and ectopic autoantigens that have limited tissue distribution (TABLE 1). In many other autoimmune diseases, autoimmunity must be elicited through immunization with tissue antigens, often in combination with adjuvants such as CFA or pertussis toxin, without evidence that similar triggers occur in the human disease counterpart. Under these non-physiological conditions, pathologies could result that have little association with the human disease. For example, concerns have recently arisen about whether data obtained from mice with experimental allergic encephalomyelitis (EAE) have been appropriately applied to the human disease that is being modelled, multiple sclerosis, and similar translational issues, as delineated in this article, will apply for T1D[102]. Alternative models, such as models of virally induced autoimmunity, should be used more frequently and when appropriate[103].

How far reaching should the conclusions be that we draw from observations in a given animal model? We suggest more caution than is usually exercised in the current scientific literature. The prevailing attitude is still that, after a discovery has been made in one of the animal models thought to represent the human disease (for example, the NOD mouse model of T1D, the SJL mouse EAE model of multiple sclerosis[104] and the (NZB × NZW)F_1 mouse model of systemic lupus erythematosus[105]), there is no urgent need to carry out similar experiments in other model systems. If the same result is found, it is considered to be confirmatory; if a different result is found, the 'secondary' model is frequently labelled as 'not as good' or even flawed, for which various reasons are then cited. We think that this can be treacherous and might hamper the translation of research carried out using animal models. An example of this is the nearly exclusive focus on the NOD mouse model. Frequently, this is because of cost considerations or lack of availability of the newer rat or transgenic mouse models. If one presumes that there is considerable heterogeneity at the population level in the pathogenesis, manifestation and clinical course of T1D, multiple sclerosis or other autoimmune diseases, then each model reflects certain aspects of pathogenesis and therefore, at best, reflects a fraction of cases. Factors such as observation for only a short period and administration of a single dose of reagent (often in a supraphysiological quantity) not only diminish the potential to strengthen statistical associations but also might eliminate the potential to uncover late therapeutic failures. So, it is highly recommended that the time period of observations in an animal model be extended before prevention is claimed.

Conclusion

Clearly, many issues require consideration when addressing what animal models can really teach us about T1D in humans. We have gained invaluable insights from research carried out so far, and it would be wrong to discount these. However, we suggest that a philosophical change in how data from interventional studies using animal models are studied, interpreted and applied would now be beneficial. In terms of experimental considerations, this would involve studies in controlled environments, interventional analyses across a broad range of times and doses, robust studies of safety, considerations of genetic and immunological differences and studies carried out in more than one animal model. Frequent comparison with emerging human data will also be necessary (including getting access to the same organs), and larger teams might be required to enable such a broad approach. Hopefully, implementing these changes will enable the lessons learned from animal models to more-rapidly find applications for the treatment and prevention of human disease.

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Of Mice and Not Men: Differences between Mouse and Human Immunology

Javier Mestas and Christopher C. W. Hughes[1]

Mice are the experimental tool of choice for the majority of immunologists and the study of their immune responses has yielded tremendous insight into the workings of the human immune system. However, as 65 million years of evolution might suggest, there are significant differences. Here we outline known discrepancies in both innate and adaptive immunity, including: balance of leukocyte subsets, defensins, Toll receptors, inducible NO synthase, the NK inhibitory receptor families Ly49 and KIR, FcR, Ig subsets, the B cell (BLNK, Btk, and λ5) and T cell (ZAP70 and common γ-chain) signaling pathway components, Thy-1, γδ T cells, cytokines and cytokine receptors, Th1/Th2 differentiation, costimulatory molecule expression and function, Ag-presenting function of endothelial cells, and chemokine and chemokine receptor expression. We also provide examples, such as multiple sclerosis and delayed-type hypersensitivity, where complex multicomponent processes differ. Such differences should be taken into account when using mice as preclinical models of human disease. *The Journal of Immunology,* 2004, 172: 2731–2738.

Mice are the mainstay of in vivo immunological experimentation and in many respects they mirror human biology remarkably well. This conservation of function is reflected in recent reports on the sequencing of both the human and mice genomes, which reveal that to date only 300 or so genes appear to be unique to one species or the other (1). Despite this conservation there exist significant differences between mice and humans in immune system development, activation, and response to challenge, in both the innate and adaptive arms. Such differences should not be surprising as the two species diverged somewhere between 65 and 75 million years ago, differ hugely in both size and lifespan, and have evolved in quite different ecological niches where widely different pathogenic challenges need to be met—after all, most of us do not live with our heads a half-inch off the ground. However, because there are so many parallels there has been a tendency to ignore differences and in many cases, perhaps, make the assumption that what is true in mice—in vivo veritas—is necessarily true in humans. By making such assumptions we run the risk of overlooking aspects of human immunology that do not occur, or cannot be modeled, in mice. Included in this subset will be differences that may preclude a successful preclinical trial in mice becoming a successful clinical trial in human.

In this review our aim is not to suggest that the mouse is an invalid model system for human biology. Clearly, with so many paradigms that translate well between the species, and with the relative ease with which mice can now be genetically manipulated, mouse models will continue to provide important information for many years to come. Rather, our aim is to sound a word of caution. As therapies for human diseases become ever more sophisticated and specifically targeted, it becomes increasingly important to understand the potential limitations of extrapolating data from mice to humans. The literature is littered with examples of therapies that work well in mice but fail to provide similar efficacy in humans (2–7). By focusing on some known differences between mouse and human immunology we hope to spur interest in this area and encourage others to note differences where they occur.

Structure and general characteristics

The overall structure of the immune system in mice and humans is quite similar. As this topic has been recently reviewed in depth (8), we will not go into great detail here. One difference worth noting is that whereas mice have significant bronchus-associated lymphoid tissue, this is largely absent in healthy humans (9), possibly reflecting a higher breathable Ag load for animals living so much closer to the ground.

The balance of lymphocytes and neutrophils in adult animals is quite different: human blood is neutrophil rich (50–70% neutrophils, 30–50% lymphocytes) whereas mouse blood has a strong preponderance of lymphocytes (75–90% lymphocytes, 10–25% neutrophils) (10). It is not clear what, if any, functional consequence this shift toward neutrophil-rich blood in humans has had.

Tyrosine kinase receptor expression on putative hemopoietic stem cells (HSC)[2] shows a reciprocal pattern, with mouse HSC being predominantly c-*kit*^{high}, *flt-3*$^{-}$, whereas human HSC are predominantly c-*kit*^{low}, *flt-3*$^{+}$ (11).

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[2] Abbreviations used in this paper: HSC, hemopoietic stem cells; iNOS, inducible NO synthase; γ_c, common γ-chain; DETC, dendritic epidermal T cells; MS, multiple sclerosis; DTH, delayed-type hypersensitivity; EC, endothelial cells.

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Innate immunity

One of the first lines of defense in higher organisms, and often the only defense in lower animals, is the growing family of antimicrobial peptides, and in particular the defensins. These are important in mucosal defense in the gut and in epithelial defense in skin and elsewhere (12, 13). Neutrophils are a rich source of leukocyte defensins in humans, but defensins are not expressed by neutrophils in mice (14). In contrast, Paneth cells, which are present in the crypts of the small intestine, express >20 defensins (cryptdins) in mice but only two in human, likely reflecting different evolutionary pressures related to microorganism exposure through food intake. There are also differences in processing of defensins (Table I).

The last few years have seen a renewed focus on the field of innate immunology, spurred in large part by identification of the Toll-like family of receptors—the TLRs (15). This field is still relatively young and so far a limited number of differences have been noted between mice and humans (Table I).

There has been considerable controversy as to whether human macrophages express NO. Expression of functional inducible NO synthase (iNOS; NOS2) in mouse macrophages has been clearly demonstrated and iNOS mRNA is readily induced by IFN-γ and LPS (16). However, these same inflammatory mediators have failed to show consistent effects on human macrophages, hence the confusion. Recent work suggests that other mediators, such as IFN-$\alpha\beta$, IL-4 plus anti-CD23, and various chemokines, are actually far more efficient in inducing iNOS in human macrophages (17). However, the controversy is not dead yet (18).

Using different strains of mice a susceptibility locus for CMV infection, *cmv1*, was identified and later shown to encode the Ly49 family of proteins (19). There are at least 14 members and most are expressed on NK and NKT cells, where the majority act as NK inhibitory receptors for MHC I molecules. The Ly49 family is absent in humans, who use the KIR family as NK inhibitory receptors (20). KIR proteins are highly diverged from the Ly49 family and have Ig rather than C-type lectin domains in their extracellular domain; however, similarly to Ly49 they also recognize MHC class I. The ligands for mouse and human NKG2D differ: in humans, NKG2D binds the polymorphic MHC class I-like molecules MHC-I chain-related A, MHC-I chain-related B, and the UL16 binding protein family, whereas in mouse NKG2D binds to H-60 and Rae1β. The significance of these differences to CMV infection and to NK biology in general have not been determined.

Adaptive immunity

FcR represent a link between the adaptive immune system, which generates Ab, and the innate immune system, which can respond to Ab-Ag complexes through capture by FcR expressed on macrophages, neutrophils, eosinophils, mast cells, and dendritic cells. There are several differences in FcR expression between mice and humans. In humans, FcαRI (CD89) is an important IgA receptor expressed by neutrophils, eosinophils, monocytes/macrophages, dendritic cells, and Kupffer cells (21). Mice lack FcαRI and presumably use alternative receptors, such as Fcα/μR, the transferrin receptor (CD71) and polymeric IgR, which also binds IgM. Humans also express two IgG receptors not found in mice: FcγRIIA and FcγRIIC are closely related single-chain FcR, each of which has a single ITAM motif in the intracellular domain. In contrast, most other FcR associate with ITAM-containing signal transduction subunits (22).

In addition to differences in FcR there are well-known differences in expression of Ig isotypes between mice and humans, and direct correlations between subtypes within classes in each species are hard to make. Mice make IgA, IgD, IgE, IgM, and four subtypes of IgG: IgG1, IgG2a, IgG2b, and IgG3. Interestingly, in the C57BL/6, C57BL/10, SJL, and NOD strains of mice there is no expression of IgG2a, instead these mice express the novel IgG2c (23). Humans in contrast express two subtypes of IgA—IgA1 and IgA2—along with single forms of IgD, IgE, and IgM. In humans there are also four subtypes of IgG: IgG1, IgG2, IgG3, and IgG4; however, these are not direct homologues of the mouse proteins. While different subtypes have differing abilities to bind FcR or fix complement, the differences between mice and humans are not considered significant. In contrast, there are differences in class switching: in mice, IL-4 induces IgG1 and IgE, whereas in humans, IL-4 induces switching to IgG4 and IgE. In contrast, IL-13 has no effect on mouse B cells but induces switching to IgE in humans (24).

There are some interesting differences in B cell development that relate to the roles of several signaling molecules. BLNK (Src homology-2 domain containing leukocyte-specific phosphoprotein-65) is an adapter protein that is rapidly phosphorylated by Syk after cross-linking of the B cell Ag receptor. It then serves as a scaffold for downstream signaling components such as Grb2, Vav, Nck, and PLC-γ. B cell development in mice lacking BLNK is blocked at the pro-B to pre-B transition, resulting in low numbers of IgM^+ B cells, but no mature $IgM^{low}IgD^{high}$ B cells, appearing in the periphery (25). A naturally occurring mutation in the human BLNK protein has been identified that results in a splicing defect preventing protein expression. In this patient there was also a block in the pro-B to pre-B transition; however, there was also a complete absence of B cells in the periphery, suggesting a more severe block in human B cell development than in mice (26).

Similarly discrepant phenotypes have been noted in mice lacking functional BCR-associated tyrosine kinase Btk (27) and in mice lacking $\lambda 5$ (28), the L chain component of the pre-BCR (Table I). Differences in mature B cells between mice and humans were recently reviewed (29), and include mutually exclusive expression of CD5 and CD23 on mouse but not human B cell subsets, and CD38 expression on human, but not mouse, plasma cells.

The discrepant phenotypes discussed above for BLNK, Btk, and $\lambda 5$ should be treated with some caution as the human diseases usually arise due to mutations in the relevant genes rather than deletions of whole exons as seen in the mouse knockout models. In some cases, however, identical mutations have been found, or created, in mice and the discrepant phenotype remains. This is the case for human XLA and mouse XID, which both involve Btk (30, 31).

The development and regulation of T cells also differs between mice and humans. Thy-1 is a GPI-linked Ig superfamily molecule of unknown function. It is expressed on thymocytes and peripheral T cells in mice and has been widely used as a T cell marker in the thymus. In humans, however, it is only expressed on neurons. The basis of this tissue specificity is suggested to be the presence or absence of an Ets-1 binding site in the third intron of the gene (32).

Table I. *Summary of some known immunological differences between mouse and human*

	Mouse	Human	Notes	Refs.
Hemotopoiesis in spleen	Active into adulthood	Ends before birth		
Presence of BALT	Significant	Largely absent in healthy tissue		9
Neutrophils in periph. blood	10–25%	50–70%		10
Lymphocytes in periph. blood	75–90%	30–50%		10
Hemotopoietic stem cells	c-*kit*^{high}, *flt-3*$^{-}$	c-*kit*^{low}, *flt-3*$^{+}$		11
TLR2 expression on PBL	Low (induced on many cells including T cells)	Constitutive (but not on T cells)	Binds lipopeptides	88
TLR3	Expressed on DC, Mac. Induced by LPS	Expressed by DC. No LPS induction	Binds dsRNA	88, 89
TLR9	Expressed on all myeloid cells, plasmacytoid DC and B cells	Expressed only on B cells, plasmacytoid DC and N	Binds CpG	90, 91
TLR10	Pseudogene	Widely expressed		
Sialic acid Neu5GC expression	Widespread	Absent	Binds pathogens	92
CD33	Expressed on granulocytes	Expressed on monocytes	Binds sialic acids	93
Leukocyte defensins	Absent	Present	neutrophils	14
Paneth cell defensins	Processed by MMP7. Stored pre-processed	Stored as pro-form. Processed by trypsin		94, 95
Paneth cell defensins	At least 20	Two		13
Macrophage NO	Induced by IFN-γ and LPS	Induced by IFN-α/β, IL-4^{+} anti-CD23		17
CD4 on macrophages	Absent	Present		96
Predominant T cells in skin and mucosa	γ/δ TCR (dendritic epidermal T cells—DETC)	α/β TCR		40
γ/δ T cells respond to phospho-antigens	No	Yes		97
CD1 genes	CD1d	CD1a,b,c,d		41
NK inhibitory Rs for MHC I	Ly49 family (except Ly49D and H)	KIR		20
NKG2D ligands	H-60, Rae1β	MIC A, MIC B, ULBP	NK activating Rs	98
fMLP receptor affinity	Low	High		99
FcαRI	Absent	Present		21
FcγRIIA, C	Absent	Present		22
Serum IgA	Mostly polymeric	Mostly monomeric		21
Ig classes	IgA, IgD, IgE, IgG1, IgG2a*, IgG2b, IgG3, IgM * absent in C57BL/6, /10, SJL and NOD mice, which have IgG2c	IgA1, IgA2, IgD, IgE, IgG1, IgG2, IgG3, IgG4, IgM		23
Ig CDR-H3 region	Shorter, less diverse	Longer, more diverse		100
BLNK deficiency	IgM^{high} B cells in periphery	No peripheral B cells		25, 26
Btk deficiency	Normal pre-B and immature B	Blocks pro-B to pre-B transition		28
λ5 deficiency	"leaky" block at pro-B to pre-B transition	Blocks pro-B to pre-B transition		28
CD38 expression on B cells	Low on GC B cells, off in plasma cells	High on GC B cells and plasma cells		29
B cell CD5 and CD23 expression	Mutually exclusive	Co-expression		29
IL-13 effect on B cells	None	Induces switch to IgE		24
Thy 1 expression	Thymocytes, peripheral T cells	Absent from all T cells, expressed on neurons		32
Effect of γ_c deficiency	Loss of T, NK, and B cells	Loss of T, NK, but B cell numbers normal		33, 34
Effect of Jak3 deficiency	Phenocopies γ_c deficiency	Phenocopies γ_c deficiency		31
Effect of IL-7R deficiency	Blocks T and B cell development	Only blocks T cell development		35, 36
ZAP70 deficiency	No CD4^{+} or CD8^{+} T cells	No CD8^{+} T but many nonfunctional CD4^{+}	Related to *syk* level?	37, 38
Caspase 8 deficiency	Embryonic lethal	Viable—immunodeficiency		62, 63
Caspase 10	Absent	Present		62
IFN-α promotes Th1 differentiation	No	Yes	Mutant stat2 in mice	44
Th expression of IL-10	Th2	Th1 and Th2		51
IL-4 and IFN-γ expression by cultured Th	Either/or	Sometimes both		
CD28 expression on T cells	On 100% of CD4^{+} and CD8^{+}	On 80% of CD4^{+}, 50% of CD8^{+}		54
ICOS deficiency	Normal B cell numbers and function, normal IgM levels	B cells immature and severely reduced in number, low IgM	Possibly age-related	55–57
B7-H3 effects on T cells	Inhibits activation	Promotes activation		101–2
ICAM3	Absent	Present	DC-SIGN ligand	103–4
P-selectin promoter	Activated by TNF and LPS	Unresponsive to inflammation		58
GlyCAM	Present	Absent		105
MHC II expression on T cells	Absent	Present		59–61
Kv1.3 K^{+} channel on T cells	Absent	Present	Regulates Ca flux	64, 65
MUC1 on T cells	Absent	Present	Regulates migration?	106
Granulysin	Absent	Present	In CTL	43

(*Table continues*)

Table I. *Continues*

	Mouse	Human	Notes	Refs.
CXCR1	Absent	Present		66, 67
IL-8, NAP-2, ITAC, MCP-4, HCC-1, HCC-2, MPIF-1, PARC, eotaxin-2/3	Absent	Present	Chemokines	66, 67
MRP-1/2, lungkine, MCP-5	Present	Absent	Chemokines	66, 67
IFN-γ effects in demyelinating disease	Protective in EAE	Exacerbates MS		4, 69–70
DTH lesions	Neutrophil-rich	Lymphocyte-rich		73, 74
Constitutive MHC II on EC	Absent	Present		80
EC present Ag to CD4+ T	No	Yes	Memory T only	75–77
CD58 (LFA-3)	Absent	Present	CD2 ligand	82
T cell dependence on CD2-ligand interactions	Low	High		82
CD2-ligand interaction	Lower affinity, with CD48	Higher affinity, with CD58		82
CD40 on EC	Absent	Present		83, 84
Vascularized grafts tolerogenic?	Yes	No		5
Microchimerism induces graft tolerance?	High success rate	Low success (expts. in non-human primates)		7
Passenger leukocytes	Account for graft immunogenicity	Do not account for graft immunogenicity		6

Similar to the development of B cells, mutation of key signaling molecules in T cells has markedly different effects in mice and humans. Several cytokine receptors, including those for IL-2, IL-4, IL-7, IL-9, and IL-15, share a common signaling chain called common γ chain (γ_c). Perhaps not surprisingly, deletion or mutation of this gene, which is on the X chromosome, results in severe immunological defects. Interestingly, these differ between human and mouse XSCID (33, 34). Numerous mutations have been identified in the human γ_c gene that inhibit function, and in most of these cases the result is a dramatic decrease in the number of T cells and NK cells. However, B cell development is normal, although function is impaired, likely due to the lack of T cell help. In marked contrast, B cell numbers are greatly diminished in γ_c-null mice. Given that IL-7R deficiency in mice blocks both T and B cell development (35), but only blocks T cell development in humans (36), it is likely that B cell development in humans is independent of IL-7. The major signal transducer for γ_c is JAK3 and mutation of this gene phenocopies the γ_c mutation in both mice and humans; that is, a lack of T and NK cells in human with the addition of a severe B cell defect in mice (31).

Interesting differences have also been noted in ZAP70-deficient mice and humans. ZAP70 is essential for TCR signaling in both developing and mature T cells, and compromised signaling results in SCID. In humans the defect results in normal numbers of $CD4^+$ T cells and absent $CD8^+$ T cells. However, the $CD4^+$ T cells are nonfunctional. In contrast, an identical mutation introduced into the mouse ZAP70 results in a block in differentiation of both T cell subsets at the double-positive stage (37). It has been suggested that the "leakiness" of the human mutant is due to incomplete down-regulation of the protein tyrosine kinase Syk in human thymocytes, compared with mouse thymocytes (38).

The study of γ/δ T cells has revealed a number of significant differences between mice and humans. T cells expressing γ/δ TCR are found in all organisms that have α/β receptors and yet their function is still largely an enigma (39). Mouse skin contains a large fraction of cells bearing a TCR encoded by a single Vγ and Vδ gene. These Vγ5-Vδ1 T cells appear to be oligoclonal, reside in the epidermis, and are known as dendritic epidermal T cells (DETC). DETC represent the predominant T cell in mouse skin, whereas cells bearing α/β receptors predominate in human skin and are found mostly in the dermis. Indeed, a cell with DETC characteristics has not been identified in humans (40). Human but not mouse γ/δ T cells have been suggested to recognize Ag presented by CD1 molecules—in particular CD1b (41). Interestingly, of the five CD1 molecules found in humans (designated CD1a, b, c, d, and e), only CD1d is expressed in mice (41). Similarly to γ/δ T cells the CD1 family of molecules has been implicated in the pathogenesis of tuberculosis, but their precise role has yet to be defined (42, 43). The differing expression of CD1 genes between mice and humans may well turn out to impact activation of both α/β and γ/δ T cells in tuberculosis, as both subsets can recognize a variety of Ags presented by CD1 molecules.

An often critical component of adaptive immunity is the skewing of T cell differentiation toward Th1 or Th2 phenotypes and this process represents another area of interaction between the innate and adaptive arms of immunity. In humans, the type I IFN, IFN-α, is secreted by several cell types in response to viral infection, including macrophages, and acts on T cells to induce Th1 development. This process is dependent upon STAT4 activation, and its recruitment to the IFN-α receptor by STAT2. In mice, however, IFN-α fails to induce Th1 cells and does not activate STAT4 (44).

The existence of polarized T cell populations was first demonstrated by Mosmann and colleagues (45) and since then has become a guiding principle for T cell activation. While polarization is relatively easy to observe in mice the paradigm has never been as clear-cut in the human system. Th1 and Th2 cells can certainly be found in human disease (46, 47); however, there is a growing recognition that in many diseases clear distinctions cannot be made and that T cells of both persuasions can often be generated simultaneously (48–50). For example, in mice, IL-10 is considered to be a Th2 cytokine, whereas in humans both Th1 and Th2 cells can make IL-10 (51). The response of mice and humans to schistosomiasis is remarkably different. Epidemiological data suggest that a Th2 response involving eosinophils and IgE may be key to combating infection in humans (52), whereas in mice effector cell activation by IFN-γ, a Th1 response, is essential for clearance of the parasite (53).

To become fully activated T cells require both a primary, Ag-dependent signal, and a second, Ag-independent or costimulatory signal. One of the best characterized costimulatory receptors is CD28, which is expressed by close to 100% of mouse $CD4^+$ and $CD8^+$ T cells. In contrast, only 80% of human $CD4^+$ and 50% of human $CD8^+$ T cells express CD28 (54), perhaps accounting for the remarkable efficacy of CTLA-4Ig in blocking T cell activation in mice. It will be interesting to see if expression of the CD28-related costimulatory molecule ICOS segregates with $CD28^-$ T cells in humans. The recent report on the identification of a human ICOS deficiency pointed to a further difference between costimulation in mice and humans. Whereas in mice the loss of ICOS does not affect either the number of mature B cells, their maturation status or their secretion of IgM (55, 56), the loss of ICOS in humans results in a severe reduction in B cell number, maturation status and secretion of IgM (57). Given the critical role of T cell CD40L in T-B interactions it would be interesting to know what the level of CD40L expression was on this patient's T cells and whether expression of this molecule is dependent upon ICOS signaling in humans. Two novel members of the B7 family of costimulatory molecules, B7-H3 and DC-SIGN, have recently also been suggested to have different roles in mice and humans (Table I).

P-selectin is constitutively expressed by endothelial cells (EC) and mediates leukocyte rolling by interactions with specific sugar residues carried by mucins. Interestingly, murine P-selectin can be strongly up-regulated by inflammatory mediators such as TNF and LPS, whereas the human gene is nonresponsive (58). It is interesting to speculate as to whether E-selectin in humans, which is strongly up-regulated by TNF, is the more important selectin on human EC for mediating leukocyte rolling.

Once activated, human T cells express MHC class II molecules whereas murine T cells do not. It has been suggested that human T cells can capture, process, and present Ag and that they express B7 and may therefore help to amplify an ongoing immune response (59, 60). In contrast, Ag presentation by T cells may also promote T cell anergy (61) or activation-induced cell death. It is not clear why this function is nonessential in mice, but it is an attractive hypothesis that it may relate to T cell homeostasis and the requirement in humans for maintaining, in a limited compartment, a greater diversity of memory T cells for a considerably longer period of time than is required in mice. T cell homeostasis requires programmed cell death (apoptosis) of unwanted cells. Caspase 8 and caspase 10 are downstream of death receptors in humans and overlap in some of their functions (62). Mice lack caspase 10 and the deletion of caspase 8 is embryonic lethal. Lack of caspase 8 in humans results in immunodeficiency, suggesting a role for this effector in lymphocyte activation as well as death (63). Greater redundancy in death receptor regulators in humans may relate to the longer lifespan and associated increased risk of developing cancer.

A critical step in activation of a T cell is the generation of a sustained calcium flux. In human T cells the inward flow of calcium ions is balance by an outward flow of K^+, mediated in large part by the Kv1.3 K^+ channel. Inhibitors of this channel very specifically block T cell activation in vitro and are being pursued as novel immunosuppressive agents (64). However, in vivo evidence to support such a function is missing as mouse T cells do not express this channel (65).

The movement of immune cells into and through tissues is coordinated by a huge array of chemokines and chemokine receptors and, not surprisingly, differences have emerged between the murine and human systems. While it is still too early to say definitively what such differences may mean, as there appears to be considerable redundancy built into the system, it is worth noting what is currently known. CXCR1 is present in humans but not in mice (66). The chemokines IL-8 (CXCL8), neutrophil-activating peptide-2 (CXCL7), IFN-inducible T cell α-chemoattractant (CXCL11), monocyte chemoattractant protein (MCP)-4 (CCL13), HCC-1 (CCL14), hemofiltrate CC chemokines-2 (CCL15), pulmonary and activation-regulated chemokine (CCL18), myeloid progenitor inhibitory factor-1 (CCL23), and eotaxin-2/3 (CCL24/CCL26) have all been identified in humans but not in mice. Conversely, CCL6, CCL9, lungkine (CXCL15), and MCP-5 (CCL12) have been identified in mice but not humans (66, 67).

Differences in immune system biology

Multiple sclerosis (MS) provides a fine example of both differences and similarities between mouse and human immunology. MS is a multifactorial disease that appears to have a large autoimmune component (68). Experimental autoimmune (allergic) encephalomyelitis is a widely used model for MS that mimics the demyelination seen in central and peripheral nerves in MS. Several studies have indicated that IFN-γ is protective in experimental autoimmune (allergic) encephalomyelitis as neutralizing Abs exacerbate disease, potentially by blocking induction/activation of suppressor activity (69, 70). It was surprising, therefore that clinical trials were not successful; indeed they were stopped because treatment with IFN-γ was found to exacerbate disease (4). In contrast, studies in mice suggested that blocking VLA-4 ($\alpha_4\beta_1$ integrin)-VCAM-1 interaction might help in MS (71) and this has indeed carried through successfully into human trials (72). These studies highlight how caution is required when extrapolating results from mouse studies to the clinic, but suggest that mouse models can successfully predict some therapies for human disease.

An interesting difference exists in the appearance of delayed-type hypersensitivity (DTH) reactions in mice and humans. In humans, around four hours after Ag challenge neutrophils can be seen forming a "cuff" around the venules. This is followed by a dramatic influx of mononuclear cells, such that by 24–48 h the lesion is mostly mononuclear with a mix of T cells and macrophages (73). Paradoxically, in mice where the peripheral blood has a relative paucity of neutrophils compared with humans, the DTH response tends to be more neutrophil rich (74). In addition, elicitation of murine DTH requires much higher concentrations of Ag than in humans.

There is now considerable evidence that human EC can present Ag to resting memory $CD4^+$ and $CD8^+$ T cells (75–77), whereas in mice, $CD8^+$ T cells can be activated by EC (78), but $CD4^+$ T cells cannot (B. Rosengard, personal communiction). As $CD4^+$ T cell-mediated activation of macrophages is thought to drive human DTH responses the suggestion has arisen that in humans, Ag transport to lymph nodes by Langerhans cells may not be necessary as EC may trigger the recall response at the site of challenge. A teleological argument can be made for the need to present Ag locally in humans but not necessarily in mice. It has been estimated that once a cell enters the lymphatics in humans it takes ~24 h to return to the

circulation if it is not retained in a node (79). Based on the higher cardiac output of mice as a proportion of their total blood volume compared with humans (5–10 ml/min, 2 ml total volume in mice; 5 L/min, 5 liter total volume in humans) it is reasonable to suppose that return of lymph is at least as fast in mice as it is in humans. Then it becomes a matter of scale. We calculate that an Ag traveling from toe to an inguinal lymph node in the groin should take ~12 h in humans and 20 min in mice. As the human DTH response begins around 4 h after secondary Ag challenge, it is possible that triggering of recall responses may occur by different mechanisms in mice and humans, involving draining of Ag to lymph nodes in mice, compared with local Ag presentation in humans.

Both human and mouse EC express MHC class I. Most human EC in vivo also constitutively express MHC class II molecules, whereas mouse EC do not (80). Thus, human EC can present Ag to $CD4^+$ T cells, as well as to $CD8^+$ T cells. A major costimulatory molecule on human EC is CD58 (LFA-3), a ligand for CD2 (81). Mice do not have the gene for CD58, which arose by CD2 gene duplication after the two lineages split. In mice the CD2 ligand is CD48; however, the distribution of this molecule differs from that of CD58 in humans, and the two-dimensional affinity for the mouse CD2-CD48 interaction is 40- to 50-fold lower than that for human CD2-CD58 interactions (82). In addition, gene deletion and Ab blocking studies have shown that mouse T cell activation is much less dependent on CD2 interactions than is the case for human T cells. Human EC also express CD40 and the ICOS ligand GL-50, whereas murine EC do not (83, 84).

The Ag presenting ability of human EC may have significant consequences for transplantation. For example, in many rodent models vascularized grafts are tolerizing, whereas such grafts are rapidly rejected in humans (5). Numerous studies have shown that purging mouse tissues of $CD45^+$ cells before transplantation dramatically extends the life of the graft, sometimes even inducing tolerance. In sharp contrast, purging human tissues of $CD45^+$ cells provides no benefit as the grafts are still rapidly rejected (6). In addition, the establishment of microchimerism in mice has been quite successful in inducing tolerance, whereas this has not been the case in humans (7). The implication of these findings is that there are major differences between mice and humans in their responses to grafted tissue, and that this may relate to the Ag-presenting ability of human, but not murine, EC.

Natural selection and the immune system

Most, if not all, of the differences we have noted between mouse and human immunology have likely become fixed during the 65 million years since our divergence because they provide some selective advantage. In all likelihood these adaptations are in response to new pathologic challenges from microorganisms, which have very short generation times and often have high mutation rates (85). In consequence, mammalian MHC molecules and NK cell inhibitory receptors have also evolved rapidly (9, 86). It should also be noted that some changes may be fixed primarily as a result of the nonimmune role of that gene—reiterative use of genes is a well recognized phenomenon during development, a good example being the important nonimmunological role of VCAM in chorioallantoic fusion and placentation (87). Thus, both the immune system as a whole, and some of its individual components (B and T cell repertoires) are shaped by natural selection.

Mice evolved in a quite different environment to humans and have been exposed to different Ags and their immune systems might therefore be expected to have evolved in subtly different ways. Mice not only live in different ecological niches, they are also much smaller and have significantly shorter lifespans. These are not trivial differences—as noted above, leukocyte transit times may be quite different in mice and humans, and a larger, broader repertoire of B and T cells must be maintained for many years in humans (up to 50 mouse lifetimes). Thus many changes may be to accommodate increased size of the organism, to regulate larger and more diverse pools of Ag-specific cells, and to provide greater checks and balances to combat the increased somatic mutation load that longer-lived animals necessarily carry.

Summary

While it is hard to draw global conclusions about the significance of differences between mouse and human immunology, it is worth considering the possibility that any given response in a mouse may not occur in precisely the same way in humans. While caution in interpreting preclinical data obtained in mice is clearly warranted, we believe that with these caveats in mind, mice will continue to be the premiere in vivo model for human immunology and will be absolutely essential for continued progress in our understanding of immune system function in health and disease.

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New Immunosuppressants: Testing and Development in Animal Models and the Clinic: with Special Reference to DSG

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INTRODUCTION

Since the start of clinical transplantation in the late '50s and early '60s the development of immunosuppressive regimens has taken two main directions, the first of which has involved the search for new immunosuppressive drugs. Experiments, mainly in rodents and dogs, have been used to discover and test new immunosuppressive drugs. The drugs azathioprine, cyclosporine A, FK506, and RS61443 have all reached the clinic because of being effective as monotherapy in experimental animals. Secondly, in conjunction with their introduction into the clinic, the drugs mentioned above have mostly been used either in combination with steroids or in various combinations one with another. Their efficacy in monotherapy has been well established but the scientific support for using these drugs in combinations has sometimes been rather weak (Starzl et al. 1963, Klintmalm et al. 1981). This is easy to understand, as the requirements demanded of clinical studies aimed at developing new immunosuppressive drugs have increased, mainly because the results are far better than in the '60s and '70s. Thus, studies require many more patients per study group to test a new immunosuppressive drug today than they did 10 years ago when cyclosporine (CyA, European multicentre trial group 1982 and Canadian multicentre study group 1985) was introduced.

The first part of this presentation will discuss the possibilities for testing new immunosuppressive drugs in a rodent model, and further to test optimal drug

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and not *in vitro* and therefore is not that well-characterized. However, LS2616 is known to stimulate both B-cell, (Spetzberg-Hagber & Larsson-Siciard 1989) T-cell (Larsson et al. 1989) and natural killer (Kalland et al. 1985) cell-mediated immune reactions. Our first experience with this substance was an experiment in which we tried to potentiate the immunosuppressive effect of cyclosporine with what we thought was a possible immunosuppressive effect of LS2616 on the survival of heterotopic rat heart allografts. Somewhat to our surprise, animals given LS2616 rejected the heart allografts at the same tempo as non-treated animals. Cyclosporin offers no protection whatsoever to the survival of the grafts (Wanders et al. 1988, see also Table I).

The unique point about this finding was that rejection took place in this rodent model despite seemingly adequate baseline immunosuppression. The dose and the administration of the drug seem to be rather uncritical. Doses from 10 mg/kg per day to 160 mg/kg per day of LS2616 seem to yield more or less the same result. The drug was easily administered in the drinking water. With one or two exceptions, in 5 years the model has never failed to produce rejection in the presence of cyclosporin A as immunosuppressant. Several rat strain combinations

TABLE I

Effect of various immunosuppressants on heterotopic cardiac graft survival in the absence or presence of LS2616 (160 mg/kg b.w.)

Group nr.	Strain	Immuno-suppression	Dose	Time	LS	Survival days	n
1	PVG-Wky	none				8	12
2	PVG-Wky	CyA	10 mg	0-9		19	5
3	PVG-Wky	CyA	10 mg	0-9	+	8	15
4	PVG-Wky	none			+	8	9
5	DAxLew-Lew	none				8	15
6	DAxLew-Lew	CyA	10 mg	0-9		22	7
7	DAxLew-Lew	CyA	10 mg	0-9	+	8	5
8	PVG-Wky	pred	15 mg	0-stop		18	8
9	PVG-Wky	pred	15 mg	0-stop	+	9	6
10	PVG-DA	none				9	9
11	PVG-DA	ATG	0.02	0		18*	10
12	PVG-DA	ATG	0.02	0	+	9	9
13	PVG-DA	none			+	8	8
14	PVG-Wky	DSG	2 mg	0-9		18	9
15	PVG-Wky	DSG	2 mg	0-9	+	13	10
16	PVG-Wky	DSG + CyA	2 mg	0-9	+	40	11
17	PVG-Wky	FK	0.3 mg	0-9	-	24	8
18	PVG-Wky	FK	0.3 mg	0-9	+	18	10
19	PVG-Wky	MC1288	0.1 μg	0-9		22	8
20	PVG-Wky	MC1288	0.1 μg	0-9	+	11	11

* 10% permanent survival. CyA given 0-stop.

have been used and it seems to work in every strain combination, except with Brown Norway as donors (an only 2-month-old result which is presently being explored). The timing of the therapy also seems to be fairly non-critical. Thus: addition of LS2616 to the recipient either several days prior to transplantation or a few days after transplantation did not alter the time of rejection, i.e. it remained at approximately 9 days. Pretreatment of the donor did not change the result.

The dose of cyclosporine was also not very critical. Doses sufficient to maintain graft survival up to sublethal doses were all totally reversed by LS2616.

Administration of higher doses of cyclosporine to a DA-to-PVG combination normally produces around 50% permanent graft survivors. The mechanism behind this permanent graft survival has been shown to be tolerance (Nagao et al. 1981). Administration of LS2616 totally abrogates the induction of tolerance. In these experiments LS2616 was given to the animals after cyclosporine therapy (Wanders et al. 1991). However, it is noteworthy that already-induced stable tolerance, i.e. animals carrying a graft permanently accepted some 60–80 days after transplantation, is not lost if LS2616 is given (Table II).

One of the first questions we raised was whether this was due to some sort of pharmacologic interaction with cyclosporine. It seems not to be: thus, when steroids were used instead of cyclosporine, exactly the same results were obtained (Table I, Gerdin 1989). Secondly, cyclosporine trough levels were sufficient to suggest that it was not due to pharmacological interaction.

Morphological and immune histochemical analyses of the grafts showed that the same cellular events take place as in untreated animals undergoing rejection.

We also used this treatment in kidney transplants, because class I-disparate rat strains do normally reject kidney grafts. LS2616 failed to induce rejection in these animals.

Based on the above-mentioned observations, we drew the conclusion that LS2616 induces rejection in CyA- or in steroid-protected grafts by a rejection mechanism not grossly different from that in non-immunosuppressed animals rejecting their grafts. The specific stimulatory effect of LS2616 seems to be to

TABLE II

Ability of LS2616 to inhibit tolerance after heterotopic cardiac transplantation

Animals	CyA-dose	Time	LS	Time (start of LS)	Tolerant animals (>100 days)
PVG-DA	20 mg	0-14			13/27
PVG-DA	20 mg	0-14	+	14-stop	0/9
PVG-DA	20 mg	0-14	+	40-stop	5/5
PVG-DA	20 mg	0-14	+	60-stop	5/5
PVG-DA	20 mg	0-14	+	90-stop	3/3*

* = > 200 days.

ments for synergism. Thus we are describing the synergistic effect of the two immunosuppressive drugs. This result has been published only recent (Gannedahl et al. 1993a) but will hopefully prompt pharmaceutical companies to test the clinical value of induction therapy with DSG.

Hopefully this type of experiment may also serve as a model to research future immunosuppressive drugs when they are going to be introduced clinically; i.e., whether they display additive or synergetic effects with CyA. Such an experimental selection procedure may somewhat reduce the otherwise almost infinite number of clinical trials needed to place a new immunosuppressive drug on the therapeutic market.

The above conclusion is, of course, only valid if the model in itself is entirely valid for the clinical situation. Until now we have had no data to suggest otherwise. It is tempting to speculate that LS induces cytokine release which induces rejection as described for IL-2 addition (Heidecke et al. 1984). However, in the long run it would be necessary to know if the effect of LS2616 is analogous to the rejection process occurring on this seemingly adequate CyA therapy. From a pragmatic point of view we have also, for the moment, nothing better to offer, and at least the procedure circumvents the problem of easy tolerance induction and absence of rejection in low levels of immunosuppressive therapy.

Xenografts as a model for anti B-cell immunosuppressive effects

One final aspect of the problem of testing immunosuppressants in small animal models has been that rodents seem to reject allografts with very low or absent B-cell responses. After immunization with cells or graft, a new graft suvives 15 days (Haltunen et al. 1987). Even LS2616 treatment produces animals which do not reject their second graft hyperacutely. That is, animals immunized with one allograft, when grafted 6 weeks later reject the second allograft at an accelerated tempo but not hyperacutely. That is, the graft rejects within 3 days. This would probably not be the situation in humans where preformed antibodies to MHC components will lead to hyperacute rejection of a second graft.

We initially found that DSG prolonged the survival of a first xenograft in a mouse-to-rat combination which is a fairly distant concordant xenograft model. (Concordant is defined as transfer of a xenograft to another species where detectable amounts of hemagglutinating antibodies are not present at the time of grafting; for review see Auchincloss 1989). Rats sensitized with the first mouse xenograft reject a second mouse xenograft (Gannedahl et al. 1992b) at the tempo of a severe hyperacute rejection, namely less than a minute. This rejection is accompanied by the presence of hemagglutinating antibodies, increased amounts of lymphocytotoxic antibodies and presence of an antibody reacting to tissue components of the heart graft as determined by immunofluorescence. Rats given DSG for 3 weeks at a dose of 5 or 10 mg/kg bodyweight do not reject the second xenograft hyperacutely. Instead the second xenograft will survive for a few hours. These animals, thus treated, do not develop the antibodies described above (Gannedahl 1993b). Thus in this experimental model it seems likely that we are testing for a desirable effect to add to the therapeutic arsenal in allografting, namely that of an anti B-cell drug. As mentioned in the introduction, rejection of a human allograft may be accompanied by the formation of antibodies.

CONCLUDING REMARKS

In the future, it would seem to be reasonable to test new immunosuppressive drugs, here examplified by DSG, in allograft models not only to show prolonged allograft survival but also to define the drug's behavior in the presence of LS2616. Moreover, one may find it possible to test new triple or quadruple or other regimens including CyA in this LS model for detection of synergy/additive effects. B-cell immunosuppressant properties may be tested in closely related concordant xenograft models.

ACKNOWLEDGMENTS

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ELSEVIER

Anti-TNFα Therapy Is Useful in Rheumatoid Arthritis and Crohn's Disease: Analysis of the Mechanism of Action Predicts Utility in Other Diseases

M. Feldman, P. Taylor, E. Paleolog, F.M. Brennan, and R.N. Maini

WHILE it is not to difficult to study the pathogenesis of animal models of disease, there are multiple constraints on analyses of the pathogenesis of human disease, leading to interesting dilemmas such as how much can we rely on and extrapolate from animal models in disease?

In the late 1980s, as a result of investigating cytokine expression and regulation in rheumatoid synovium, both in vitro and in vivo, we found that tumor necrosis factor (TNF)α was of major importance, as it regulated the expression of the other proinflammatory cytokine interleukin-1 (IL-1).[1] We proposed that blocking TNFα would thus have major effects on the inflammatory process and hence would be clinically useful.[2] That prediction has been substantiated in clinical trials that we have led using Centocor's chimaeric monoclonal antibody, cA2, now termed Remicaide. Positive results were also reported using a humanized antibody produced by Celltech[3] and also using a TNF-receptor p75 IgGFc fusion protein, produced by Immunex, termed Enbrel.[4] Clinical effects of anti-TNFα have recently been reviewed.[5]

RESULTS AND DISCUSSION

While the clinical results with the above three biologic therapeutic agents have been in the same ball park, mechanism of action studies reported are only with cA2. The antibodies differ from TNF-R p75Fc in that the latter also blocks the related protein lymphotoxin, while the former are specific to TNFα. The consequences of this difference are not known, but differences are conceivable.

The first pathogenic mechanisms that occur to be clarified in rheumatoid arthritis was the existence of a cytokine cascade in vivo, as had been described in vitro. The effect was rapid diminution of serum IL-6, and other cytokine levels.[5,6] This accounts, in part, for the rapid onset of beneficial effects seen locally. Equally interesting was the observation that levels of cytokine inhibitors such as IL-1ra and soluble TNF-R are also diminished rapidly. The latter observations confirm that both the proinflammatory and antiinflammatory arms of the cytokine cascade are TNFα dependent. This is discussed in detail elsewhere.[5,6] In rheumatoid synovial cultures in vitro the effect of anti TNFα antibody occurred subsequent to TNFα neutralization. In vivo, there is the interesting possibility that some of the cells producing TNFα which have TNFα on their surface prior to it being cleaved by "TNFα convertase," may be killed by the antibody in the presence of complement from serum or by antibody-dependent cell-mediated cytotoxicity. Such events can be shown in vitro.[7]

Recruitment of leucocytes to inflammatory sites is an essential step in permitting the inflammation to develop. This is a complex process, involving both adhesion molecules of various families (selectins, integrins, and so forth) as well as chemotactic factors, with chemokines being the most abundant. It was found that cA2 therapy diminished the expression of adhesion molecules E selectin, ICAM-1, and VCAM-1 in various assays, and that chemokine production such as IL-8 and MCP-1 was reduced, indicating downregulation of parameters for leucocyte recruitment.[8] Formal proof of diminished trafficking has come from studies with radiolabeled leucocytes (Taylor et al, in preparation).

Synovium in chronic rhematoid arthritis is very vascular, and to support the mass of synovium, angiogenesis is required. Anti-TNFα antibody therapy was found to downregulate the levels of vascular endothelial growth factor, suggesting that angiogenesis may also be regulated by the inflammatory response. Support for that concept was also found in vitro.[8]

The conclusion from these studies is that in a chronic immune-driven inflammatory response there are a number of pathways that become engaged and can serve to sustain the inflammatory process. Those delineated above are not necessarily the only ones.

IMPLICATIONS FOR OTHER DISEASES

Local recruitment of leucocytes to the disease site occurs in many diseases. It is regulated by the same families of adhesion molecules and chemokines as in rheumatoid

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arthritis. It is thus likely that in many diseases, TNFα is involved in the recruitment phase, and hence blocking TNFα early in the disease process may be an effective way of reducing the mass of infiltrating cells causing inflammatory damage.

The linkage of the regulation of both proinflammatory and antiinflammatory mediators suggests that the most effective therapy in immune inflammatory diseases such as rheumatoid arthritis, Crohn's, and multiple sclerosis will come from therapy aimed at several points in the disease pathway. In the animal model of arthritis (collagen induced arthritis) we have shown that there is synergy of anti-TNFα therapy with anti-T-cell therapy such as lytic anti CD4 antibodies.[9] It is possible that such combination therapies, if given early in the course of the disease process, may be able to control the disease.

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Promotion of Pancreatic Islet Allograft Survival by Intrathymic Transplantation of Bone Marrow

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An important goal in the treatment of insulin-dependent diabetes by pancreatic islet transplantation is the development of strategies that allow permanent survival of islet allografts without continuous host immunosuppression. In this study, we demonstrate that inoculation of allogeneic bone marrow into the thymus of adult rats treated with a single dose of anti-lymphocyte serum induces an unresponsive state that permits survival of subsequent pancreatic islet allografts transplanted to an extrathymic site. This effect is donor specific, cannot be reproduced by systemic administration of bone marrow, and is associated with persistence of chimeric cells in the thymus of the recipient. In addition, lymph node cells from long-term recipients of intrathymic bone marrow display markedly reduced proliferative responses to donor alloantigens in mixed lymphocyte culture. Interaction of maturing thymocytes with foreign alloantigens may produce the unresponsiveness. This model offers a potential approach for establishing donor-specific allograft acceptance in adult recipients. *Diabetes* 41:771–75, 1992

Induction of donor-specific unresponsiveness represents the ideal approach for securing permanent survival of pancreatic islet allografts because it precludes rejection without the need for chronic immunosuppression of the host. Although it was demonstrated >30 yr ago that immunologic tolerance can be readily achieved in rodents by inoculation of donor-strain lymphohematopoietic cells at birth, tolerance induction in adult recipients has been more difficult, requiring extensive preparative conditioning by irradiation and/or treatment with nonspecific cytoablative chemotherapy (1–4). We previously reported that pancreatic islets implanted in the thymus of allogeneic adult rats survived permanently and, in addition, rendered the recipients tolerant of donor alloantigens (5). In this study, the efficacy of this approach in promoting transplantation tolerance was assessed by examining the impact of intrathymic inoculation of allogeneic bone marrow cells (BMC) on the survival of extrathymic transplants of pancreatic islets. We demonstrate that rats pretreated with an intrathymic injection of allogeneic BMC are rendered specifically unresponsive to donor alloantigens and permanently accept subsequent donor-strain islet allografts. Furthermore, this result can be accomplished without need for other methods known to prolong islet allograft survival, such as pretransplant modulation of allograft immunogenicity or chronic immunosuppression of the recipient (6).

RESEARCH DESIGN AND METHODS

Bone marrow inoculation and islet transplantation. BMC were obtained from adult male Lewis ($RT1^l$) donors, depleted of contaminating erythrocytes by centrifugation on a Ficoll-isopaque gradient and inoculated intravenously into an abdominally displaced testicle or into both thymic lobes of histoincompatible male Wistar Furth (WF) ($RT1^u$) recipients. Each recipient received $60–70 \times 10^6$ nucleated cells; rats given intravenous BMC inocula received intrathymic injections of saline. Where noted, recipients were treated with a single dose of 1 cc rabbit anti-rat lymphocyte serum (ALS) i.p. (Accurate Chemical and Scientific, Westbury, NY) on the day of BMC injection. No additional immunosuppression was administered at any other time during the experiment. The rats were then rendered diabetic with 65 mg/kg streptozocin i.v. and received freshly isolated (uncultured) Lewis or DA ($RT1^a$) islet allografts beneath the renal capsule 14 days after BMC inoculation. Only rats with nonfasting blood glucose levels >300 mg/dl

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TABLE 1
Survival of Lewis islet allografts in Wistar Furth recipients

Group	Site of Lewis bone marrow cells inoculation*	Anti-rat lymphocyte serum treatment†	Individual graft survival (days)
1	None	None	9, 9, 10, 13 (9.5)
2	None	+	8, 9, 14, 15, 18, >173‡ (14.5)
3	Intravenous	+	13, 16, 21, 23, 32, 32 (22)§
4	Thymus	+	12, 28, >130, >130, >148, >159, >183 (>130)‖
5	Testicle	+	7, 8, 8, 10 (8)

Values in parentheses are median survival times.
*Administered 14 days before islet transplantation.
†One cubic centimeter given intraperitoneally at time of bone marrow cell injection.
‡Rat reverted to hyperglycemia after removal of islet-bearing kidney.
§$P > 0.1$ vs. group 2.
‖$P < 0.04$ vs. groups 2 or 3.

were used as recipients. Islets were isolated as previously described (7). Graft survival was monitored by daily blood glucose measurements, with rejection being defined as a return to hyperglycemia (blood glucose ≥200 mg/dl on 2 consecutive days). In recipients with persistent normoglycemia, islet allograft function was eventually confirmed by removal of the islet-bearing kidney for histological examination.

Immunohistochemistry. To detect the presence of donor-strain cells in WF rats inoculated with Lewis BMC, acetone-fixed frozen sections (5–6 μm) of thymus, peripheral lymph nodes, and testicle were incubated overnight at 4°C with culture supernatant containing a murine monoclonal antibody (I-1.69) specific for Lewis class I MHC antigens (RT1-A^1) (8). Sections were then exposed to biotinylated horse anti-mouse IgG followed by avidin-conjugated peroxidase according to manufacturer's instructions (Vector Laboratories, Burlingame, CA). Conjugates were visualized with diaminobenzidine hydrochloride and lightly counterstained with methyl green before mounting. Control sections incubated without specific primary antibody did not show any background staining.

Mixed lymphocyte culture (MLC). Graded numbers of lymph node (LN) cell responders ($0.75–3 \times 10^5$/well) were cultured at 37°C with 2000R irradiated LN cell stimulators (3×10^5/well) in 96-well flat-bottom plates containing RPM1–1640 supplemented with 5% fetal calf serum, 2-mercaptoethanol, 1-glutamine, and antibiotics. Plates were pulsed with ^{3}H-thymidine (1 μCi/well) on day 3 and harvested 24 h later.

Statistics. Statistical differences between treatment groups were determined with Wilcoxon's rank-sum test. $P < 0.05$ was significant.

RESULTS AND DISCUSSION

Normal WF recipients that were not pretreated with intrathymic Lewis BMC or ALS rapidly rejected the Lewis islet grafts (median survival time [MST] 9.5 days; Table 1). Five of six WF recipients that received intrathymic injections of saline in conjunction with ALS 2 wk before islet transplantation also promptly rejected Lewis islet allografts (MST 14.5 days), although one rat remained normoglycemic for >170 days. Intravenous injection of Lewis BMC in conjunction with ALS 2 wk before islet grafting led to modest prolongation of Lewis islet allograft survival (MST 22 days); however, all allografts eventually underwent rejection within 32 days. In contrast to all of these control groups, when Lewis BMC were administered intrathymically, five of seven WF recipients of Lewis islet allografts remained permanently normoglycemic. Histological examination of the grafts at the conclusion of the experimental period revealed numerous clusters of well-granulated islets with no evidence of intra-islet mononuclear cell infiltration (Fig. 1, *A* and *B*). The specificity of the tolerant state induced by inoculation of Lewis BMC was assessed by grafting WF rats that had received intrathymic or intravenous Lewis BMC with pancreatic islets from DA (RT1^a) donors. These third-party grafts were all rejected within 10 days (Table 2).

Prolonged residence of allogeneic tissue in an immunologically privileged site weakens the host's immune responsiveness to subsequent donor-strain allografts transplanted to conventional sites (9,10). Because the thymic parenchyma is relatively inaccessible to the peripheral immune system (11), it was conceivable that the unresponsiveness observed in intrathymically treated rats was solely due to the presence of the conditioning BMC inoculum and that the special immunologic functions of the thymus were not relevant (i.e., its role in T-cell maturation and induction of self-tolerance) (12). To evaluate this possibility, we implanted allogeneic Lewis BMC into another immunologically privileged site, the abdominally displaced testicle, of WF recipients given a single injection of ALS (9). Two weeks after intratesticular BMC inoculation, these recipients were rendered diabetic and transplanted with Lewis islet allografts. All rats rapidly rejected the islets (MST 8 days), indicating that the protective influence of the intrathymic bone marrow inoculum on subsequent allografts was unlikely to be explained entirely by its inoculation in a privileged site (Table 1).

In accordance with the in vivo findings, intrathymic inoculation of allogeneic Lewis BMC had a marked influence on T-cell–mediated responses to donor alloantigens in vitro. LN cells from recipients in which long-term Lewis islet allograft survival was achieved by intrathymic BMC inoculation responded normally to third-party DA stimulators in an MLC system; however, proliferation of

FIG. 1. Photomicrographs of kidney and thymus from Wistar Furth (WF) recipients of Lewis bone marrow cells (BMC) and pancreatic islet allografts. *A*: kidney from a WF rat examined 160 days after intrathymic Lewis BMC inoculation and subsequent transplantation of Lewis islets beneath the renal capsule. The islet graft (*arrows*) appears healthy and shows no infiltration by mononuclear cells (hematoxylin-eosin, ×100). *B*: the presence of β-cells in the same section is verified by staining for insulin granules (aldehyde-fuchsin, ×100). *C–E*: immunoperoxidase localization of Lewis ($RT1^1$) cells in the thymus of WF recipients of Lewis BMC: *C*: thymus from a rat killed 14 days after intrathymic BMC inoculation. Numerous donor-strain cells are scattered throughout the thymic parenchyma (×80). *D*: thymus from the rat depicted in *A* demonstrating persistence of Lewis cells 160 days after inoculation (×100). *E*: thymectomy specimen from a WF rat given intravenous injection of Lewis BMC 14 days previously. No $RT1\text{-}A^1$–specific staining is detectable (×80).

these cells to Lewis stimulators was consistently decreased compared with responses of unmanipulated controls (Fig. 2). Because proliferation of rat lymphocytes in primary MLC predominantly reflects activity of class II MHC-restricted $CD4^+$ T cells (13,14), these results suggest that the tolerant state at least in part affects this cell population. Interestingly, we previously found that in WF rats bearing long-term intrathymic Lewis islets, MLC responses to Lewis alloantigens were not reduced compared with unmanipulated controls (5). One explanation for this difference is that in contrast to bone marrow, which consists of 5–10% class II MHC^+ cells and con-

TABLE 2
Survival of DA islet allografts in Wistar Furth rats

Site of Lewis bone marrow cells inoculation*	Individual graft survival (days)
Intravenous	7, 8, 8, 8 (8)
Thymus	7, 8, 8, 10 (8)

Values in parentheses are median survival times.
*Administered in conjunction with 1 cc anti-rat lymphocyte serum i.p. 14 days before islet transplantation.

tains progenitors capable of differentiating into populations that constitutively express high levels of class II MHC antigens (dendritic cells, macrophages, B cells), class II expression on islets is limited to rare intra-islet dendritic cells (15,16). Consequently, the residence of islets in the thymic microenvironment may not measurably affect class II–restricted in vitro responses.

In several models of specific unresponsiveness achieved by allogeneic BMC transplantation, tolerance correlates with the presence of microchimerism in the thymus and peripheral lymphoid organs of the recipients (17–19). To determine whether a similar chimeric state had developed in our model, the lymphoid organs of WF rats that had received intrathymic Lewis BMC (without subsequent islet grafts) were examined at various times after inoculation by immunohistochemistry with a murine monoclonal antibody (I-1.69) specific for Lewis class I alloantigens (8). Although chimerism was not observed in the LN of these rats at any time after intrathymic injection, donor-strain cells were uniformly detected in thymic sections from rats inoculated with Lewis BMC 2–45 days earlier. Similarly, in three of four rats that had accepted Lewis islet grafts after conditioning with intrathymic Lewis BMC, donor-strain cells were found in the thymus when it was examined 140–170 days after inoculation, although the number of cells was reduced compared to organs examined at earlier time points (Fig. 1, *C–E*). In contrast, donor-strain cells were not detected in the thymus, LN, or testicles of rats given intravenous or intratesticular BMC. Thus, it appears that the thymic microenvironment can support the long-term survival of implanted hematopoietic cells and is capable of protecting them (or their descendants) from elimination by immune mechanisms.

Our findings demonstrate that intrathymic inoculation of allogeneic BMC is an effective means by which specific unresponsiveness to pancreatic islet allografts can be achieved. Significantly, we show that this tolerant state develops relatively soon after thymic injection and, as demonstrated by the prompt rejection of grafts by rats given intravenous or intratesticular BMC, requires that the allogeneic inoculum is situated in the thymic microenvironment.

Several experimental systems have shown that intrathymic inoculation of foreign substances can have a profound impact on the systemic immune responsiveness of the recipient. Staples et al. (20) were the first to report that irradiated rats given intrathymic injections of bovine γ-globulin displayed markedly reduced antibody synthesis and delayed-type hypersensitivity responses to the antigen (20). More recently, it was demonstrated that intrathymic injection of allogeneic $CD4^-/CD8^-$ prothymocytes, B cells, or dendritic cells could specifically reduce T-cell–mediated responses to donor antigens, an effect attributed to intrathymic deletion or functional inactivation of donor-specific clones (21,22). A similar mechanism could be invoked to explain the tolerant state observed in this study. Intrathymic inoculation of allogeneic BMC may provide a particularly efficient means of establishing a state of chimerism in the thymus and promoting exposure of maturing thymocytes to donor alloantigens. The resulting interactions may then lead to selective deletion or functional inactivation of donor-specific clones before their migration to the periphery. Because the allogeneic inoculum is introduced at a time when the peripheral T-cell pool has been transiently depleted by ALS, this effect would be expected to have a maximal influence on the reconstituting T-cell repertoire. In addition, the relative immunologic privilege of the thymic parenchyma may protect implanted cells from destruction by alloimmune processes, thus enabling them to influence T-cell development for an extended period of time.

Our studies demonstrate that intrathymic transfer of allogeneic cells in conjunction with a single dose of ALS can promote long-term survival of subsequently transplanted islet allografts. This model provides a novel strategy for the induction of specific unresponsiveness in adult rats and may lead to the development of methods that permit long-term allograft survival without the need for prolonged, nonspecific immunosuppression of the host.

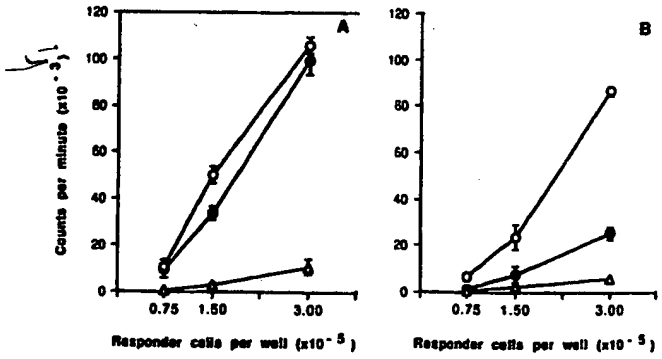

FIG. 2. Proliferative responses of lymph node cells from Wistar Furth controls (*A*) and Wistar Furth recipients in which long-term (>130 days) Lewis islet allograft survival was achieved by intrathymic inoculation of Lewis bone marrow cells (*B*). Graded numbers of responder cells were cultured alone (△), with irradiated Lewis (●), or with DA (○) lymph node stimulators. Values are means ± SD of triplicate cultures of 3 similar experiments.

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Belyavskyi	
ART UNIT	PAPER NUMBER

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INTERVIEW SUMMARY

All participants (applicant, applicant's representative, PTO personnel):

(1) Belyavskyi
(2) Latter Paul
(3) Marina Gordey
(4) Kirk Hahn
(5) David Aliner

Date of Interview 3/21/05

Type: ☐ Telephonic ☐ Televideo Conference ☒ Personal (copy is given to ☒ applicant ☐ applicant's representative).

Exhibit shown or demonstration conducted: ☐ Yes ☐ No If yes, brief description: ____

Agreement ☐ was reached. ☒ was not reached.

Claim(s) discussed: ALL

Identification of prior art discussed: ____

Description of the general nature of what was agreed to if an agreement was reached, or any other comments:

Applicant will provide Declaration showing the histology of the mice that were presented in type I diabetic. Applicant will consider to amend claims to specifically recite the claim invention. The Examiner provided the correct copy of Vossel et al

(A fuller description, if necessary, and a copy of the amendments, if available, which the examiner agreed would render the claims allowable must be attached. Also, where no copy of the amendments which would render the claims allowable is available, a summary thereof must be attached.)

☒ It is not necessary for applicant to provide a separate record of the substance of the interview.

Unless the paragraph above has been checked to indicate to the contrary. A FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION IS NOT WAIVED AND MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has are ready been filed, APPLICANT IS GIVEN ONE MONTH FROM THIS INTERVIEW DATE TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW.

Examiner Note: You must sign this form unless it is an attachment to another form. [signature]

Intrathymic Islet Transplantation in the Spontaneously Diabetic BB Rat

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Recently it was demonstrated that pancreatic islet allografts transplanted to the thymus of rats made diabetic chemically are not rejected and induce specific unresponsiveness to subsequent extrathymic transplants. The authors report that the thymus can also serve as an effective islet transplantation site in spontaneously diabetic BB rats, in which autoimmunity and rejection can destroy islets. Intrathymic Lewis islet grafts consistently reversed hyperglycemia for more than 120 days in these rats, and in three of four recipients the grafts promoted subsequent survival of intraportal islets. In contrast intraportal islet allografts in naive BB hosts all failed rapidly. The authors also show that the immunologically privileged status of the thymus cannot prevent rejection of islet allografts in Wistar Furth (WF) rats sensitized with donor strain skin and that suppressor cells are not likely to contribute to the unresponsive state because adoptive transfer of spleen cells from WF rats bearing established intrathymic Lewis islets fails to prolong islet allograft survival in secondary hosts.

THE FAILURE OF currently available immunosuppressive protocols to prolong the survival of pancreatic islet allografts has prompted a search for alternate methods that can permit long-term graft function without the need for chronic immunosuppression. One strategy used with some success in animal systems is the transplantation of endocrine tissue to natural or artificially constructed immunologically privileged sites. While many of the best described privileged sites, such as the anterior chamber of the eye and alymphatic skin flap, are incapable of supporting islet endocrine function, others, such as the cerebral cortex and abdominally displaced testicle, have been shown to permit survival of implanted islets and in addition to protect them from alloimmune rejection.[1-3] Although these techniques deserve consideration because they obviate the need for host immunosuppression or pretransplant immunomodulation of the graft, practical considerations would limit their use in clinical islet transplantation.

Recently we reported that the thymus can serve as a novel transplant site that provides a suitable environment for islet endocrine function and promotes survival of allografts without chronic immunosuppression of the recipient. Furthermore animals bearing established intrathymic islet grafts are rendered specifically unresponsive to donor alloantigens as demonstrated by their inability to reject donor-strain islets transplanted to an extrathymic site.[4]

In the present study, we have evaluated further the mechanisms responsible for prolonged survival of intrathymic grafts and for the induction of specific tolerance that follows intrathymic islet transplantation. In addition we have determined the outcome of intrathymic islet transplantation in spontaneously diabetic BB rats, in which both rejection and recurrent anti-beta cell autoimmunity can destroy islet allografts.[5-7]

Materials and Methods

Animals

BB rats were bred and maintained in the animal facilities of the University of Pennsylvania. In our subline of diabetes-prone rats, spontaneous diabetes has a cumulative incidence of 50% to 60% and develops between 60 to 120 days of age. Rats that were considered to have

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developed diabetes (serum glucose levels more than 300 mg/dL on 3 consecutive days) received daily injections of protamine zinc insulin to prevent ketoacidosis and death. Insulin therapy was discontinued after islet transplantation so that blood glucose levels could serve as an index of islet graft survival. Spontaneous recovery from diabetes in BB rats has never occurred in our colony. All BB rats are homozygous for the $RT1^u$ haplotype at the major histocompatibility complex (MHC) locus.

Inbred Wistar Furth (WF, $RT1^u$) and Lewis ($RT1^l$) rats were purchased from Harlan-Sprague Dawley (Walkersville, MD) and Charles River Laboratories (Wilmington, MA), respectively.

Induction of Chemical Diabetes

Hyperglycemia was induced in nondiabetic WF rats by a single intravenous injection (65 mg/kg) of streptozotocin. Only rats with nonfasting blood glucose levels of more than 300 mg/dL were considered suitable for transplantation with pancreatic islets.

Islet Isolation and Transplantation

Islets were isolated by collagenase digestion of the pancreas followed by centrifugation through a discontinuous Ficoll gradient as previously described.[8] Contaminating nonislet tissue was removed under a dissecting microscope, and only islets free of adherent acinar, vascular, and lymphoid elements were used for transplantation. Spontaneously diabetic animals received islet grafts 2 to 7 days after onset of diabetes, while chemically induced diabetic rats were allowed to remain hyperglycemic for 6 to 14 days to exclude the possibility that insufficient streptozotocin had been administered to cause permanent diabetes. Freshly isolated islets (1000 to 1500) were transplanted into the following sites: (1) the liver *via* portal vein embolization, (2) the renal subcapsule, (3) the abdominally displaced testicle, and (4) the thymus by injection of 600 to 800 islets into each lobe. Islets were never maintained in tissue culture before transplantation. Hyperglycemia usually was reversed within 1 day and always within 4 days after transplantation. Where noted recipients received 1 mL of rabbit anti-rat lymphocyte serum (ALS) (Accurate Chemical and Scientific Co., Westbury, NY) intraperitoneally at the time of transplantation. Nonfasting blood glucose levels were determined three times each week in all islet recipients, with cessation of graft function being defined as the first of 2 consecutive days of recurrent hyperglycemia (blood glucose level more than 200 mg/dL). At the conclusion of each study, animals were killed and islet-bearing organs were removed, fixed in Bouin's solution, and processed for light microscopy. Serial sections of each organ were stained with hematoxylin and eosin as well as with aldehyde-fuchsin for identification of islet tissue.

Monoclonal Antibodies (MAb) and Flow Cytometry

The following murine mAbs specific for rat lymphocyte markers were used: R73 (anti-α/β T-cell receptor [TCR]; all T cells), OX-8 (anti-CD8; cytolytic/suppressor T cells and NK cells), W3/25 (anti-CD4; helper T cells and macrophages), and Mar 18.5 (anti-surface immunoglobulin, (sIg); B cells). The antibodies were characterized previously.[9,10] Monoclonal reagents were used either as undiluted culture supernatants or obtained in purified form from Bioproducts for Science, Inc. (Indianapolis, IN).

For immunofluorescent analysis, 1 to 2×10^6 cervical and mesenteric lymph node cells (LNC) were incubated for 60 minutes with saturating concentrations of the primary mAb, washed twice, and treated for 60 minutes with fluorescein isothiocyanate-conjugated $F(ab')_2$ goat anti-mouse IgG (Tago, Inc., Burlingame, CA). All incubations were performed at 4 C in Dulbecco's phosphate-buffered saline (D-PBS) containing 0.01% sodium azide. Ten thousand viable cells were analyzed for relative fluorescence using a dual laser fluorescence-activated cell sorter (FACS) with logarithmic amplifiers (FACS IV; Becton-Dickinson, Sunnyvale, CA). Background fluorescence was calculated using cells incubated with the fluorescein conjugate alone.

Adoptive Transfer Studies

Spleens from long-term (more than 200 days) WF recipients of intrathymic Lewis islet allografts were teased into single-cell suspensions, washed twice in D-PBS, and injected intravenously (250 to 300 $\times$ 10^6 cells/recipient) into sublethally irradiated (450 R), streptozotocin-diabetic WF rats. Twenty-four hours later, animals received fresh Lewis islet allografts beneath the renal capsule without immunosuppression. Control animals received spleen cells from unmanipulated WF rats.

Skin Grafting

Skin grafting was performed according to the method of Billingham.[11] Rejection was defined by extensive graft necrosis and sloughing, as judged by daily inspection after dressings were removed on the seventh day.

Results

Survival of Islet Allografts in Nonimmunosuppressed Spontaneously Diabetic BB Rats

Pancreatic islets isolated from Lewis donors and transplanted by portal vein inoculation into six spontaneously diabetic BB rats were destroyed promptly in every instance (median survival time [MST], 9 days). In this model it is

1,000 1,500 islets × 1000 cells/rat produced normoglycemia

140 (250 g)

TABLE 1. *Survival of MHC-compatible and -incompatible Islet Allografts in BB Rats*

Transplant Site	Donor Strain (MHC)	Days of Allograft Survival (MST)*
Liver (intraportal)	Lewis ($RT1^l$)	8, 9, 9, 19, 24, (9)*
Renal subcapsule	Lewis ($RT1^l$)	41, 47, 59, >70† >120 × 2 (>64.5)
	WF ($RT1^u$)	23, 64, >120 (64)
Thymus	Lewis ($RT1^l$)	>50 × 5, >120 × 6 (>120)‡
	WF ($RT1^u$)	>120 × 5 (>120)

impossible to distinguish rejection from autoimmune damage, which in itself can cause islet failure. As we have found previously, islets transplanted beneath the renal capsule had a more prolonged and variable survival in BB rats, which are known to be significantly immunodeficient.[12,13] Of the 6 recipients of renal subcapsular grafts, 3 underwent islet failure after 41, 47, and 59 days, 1 died while still normoglycemic after 70 days, and 2 others remained normoglycemic for more than 120 days before they were used for other experiments (see below). In contrast to the results in other transplant sites, none of 11 BB rats that received intrathymic Lewis islets destroyed their grafts. Serum glucose levels in these animals returned to normal within 48 to 72 hours after islet transplantation and remained between 80 and 110 mg/dL for the duration of the study. Six of these animals, after being followed for more than 120 days, were then studied histologically and/or used for other experiments. Five other normoglycemic animals are still being followed for periods between 50 and 67 days. These findings are summarized in Table 1.

If autoimmune diabetes is an MHC-restricted process, islet allografts from MHC-compatible donors, although less susceptible to rejection by BB recipients than those from MHC-incompatible donors, would be more likely to be destroyed by autoimmunity.[14,15] Therefore to investigate whether the thymus protects MHC-compatible islet grafts from rejection and, more importantly, also from autoimmune damage, we compared the outcome of intrathymic and renal subcapsular grafts of WF islets in spontaneously diabetic BB rats. All five recipients of intrathymic WF islets maintained normal serum glucose levels for more than 120 days, while two of three recipients of renal subcapsular WF grafts became hyperglycemic after 23 and 64 days (Table 1).

Assessment of Unresponsiveness in Islet Allograft Recipients

To determine whether intrathymic islets transplanted to diabetic BB rats would induce an unresponsive state that could protect subsequent extrathymic islets from either rejection or autoimmunity, five BB rats that had harbored an intrathymic Lewis islet transplant for 120 days received another Lewis islet allograft transplanted intraportally. No immunosuppression was given at any time. All animals remained normoglycemic and 60 to 65 days after the second allograft the thymus containing the first allograft was removed in four of these rats to allow functional assessment of the extrathymic islet transplant (Fig. 1). Three of the four animals remained normoglycemic after thymectomy while one (BB#1) became hyperglycemic (blood glucose level, 250 to 300 mg/dL) although it remained healthy and continued to gain weight, unlike

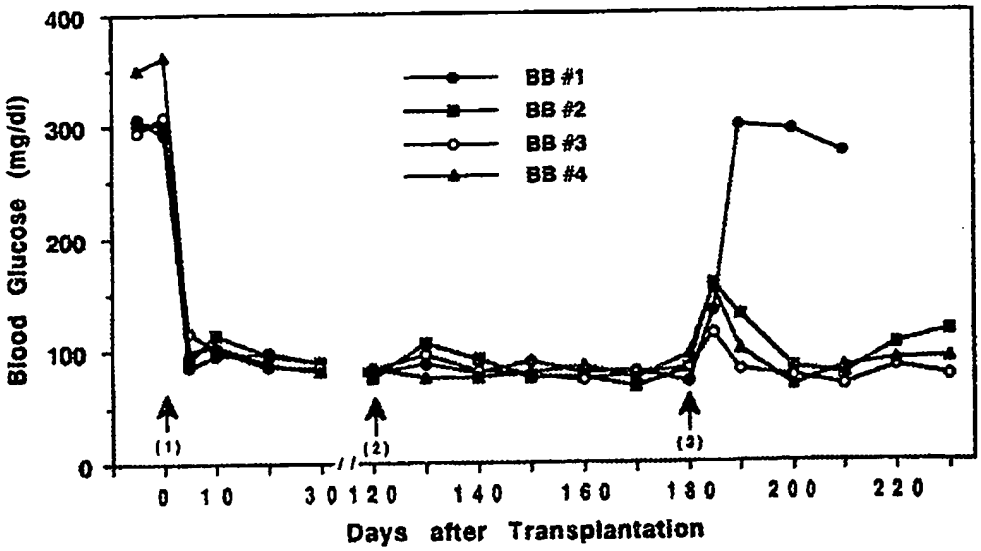

FIG. 1. Blood glucose profiles of spontaneously diabetic BB rats (BB # 1-4) transplanted with Lewis islet allografts. (1) Intrathymic islet transplantation; (2) second transplantation of Lewis islets intraportally; (3) removal of islet-bearing thymus.

untreated BB rats, which usually do not survive without exogenous insulin treatment.

In all six recipients of intrathymic Lewis islets, histologic examination of the thymus after 120 days revealed healthy, well-granulated islets and no lymphocytic infiltrate. In the animals that had remained normoglycemic after thymectomy, the liver, when finally examined histologically, also contained well-granulated noninfiltrated islets (Fig. 2). The liver of the one rat that became hyperglycemic after thymectomy contained intact islets with

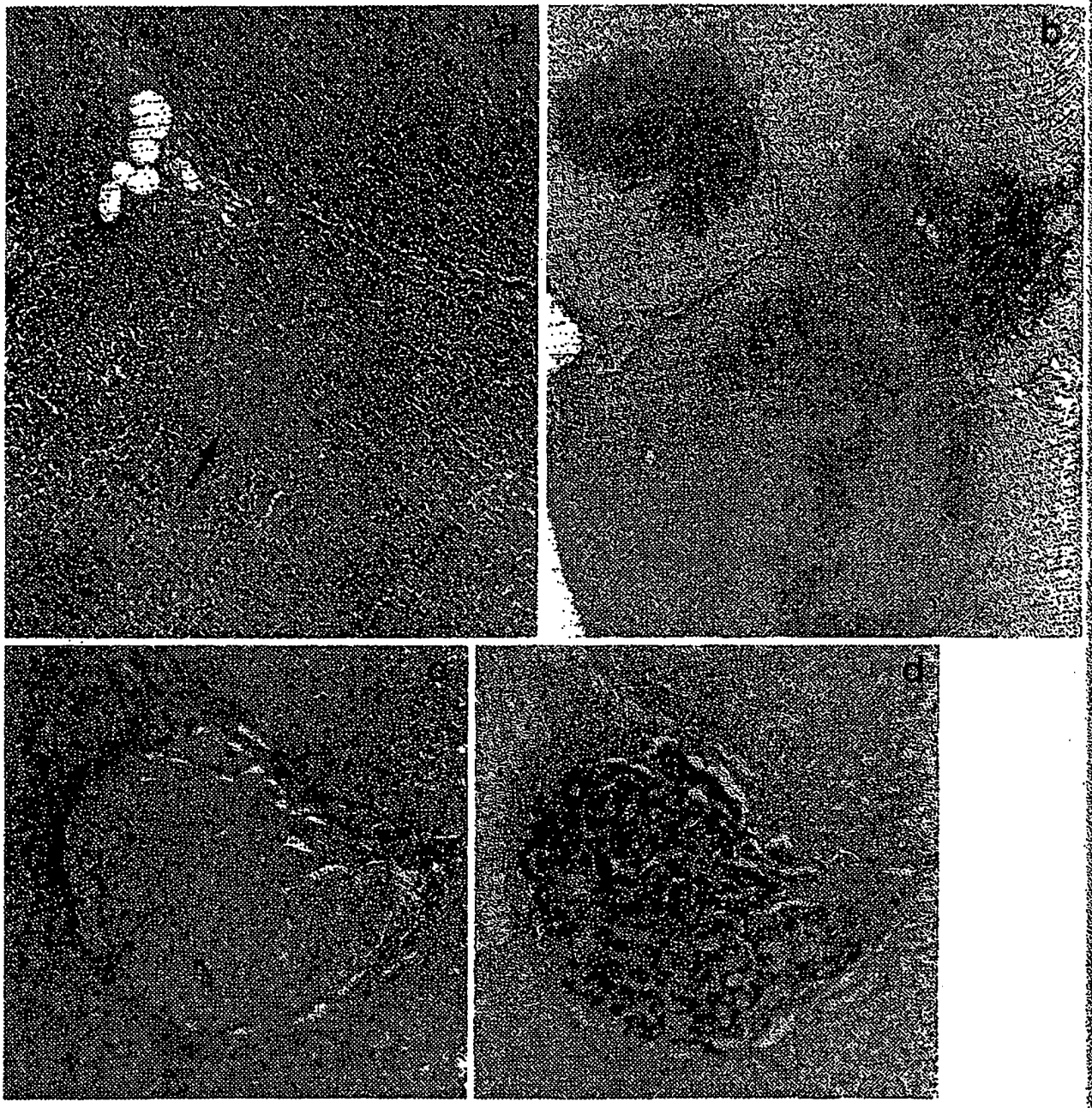

FIG. 2. Photomicrographs of thymus and liver from BB recipients of Lewis islet allografts. (a) Section of a thymus removed 180 days after implantation of Lewis islets. The islets (arrow) appear healthy and there is no mononuclear infiltration (H&E ×80). (b) Aldehyde-fuchsin–stained section from the same specimen demonstrating abundant insulin granules (×20). (c) Section of liver from a rat that was transplanted with intraportal Lewis islets 120 days after intrathymic islet transplantation. Removal of the liver was performed 110 days after intraportal islet transplantation (H&E ×160). (d) The presence of beta cells in the same section is indicated by insulin-specific staining (aldehyde-fuchsin ×160).

no suggestion of insulitis; however they stained weakly for insulin. This histologic finding suggested that an insufficient number of islets had been transplanted to the liver to allow the intraportal graft alone to maintain normoglycemia after removal of the thymus. Such grafts are likely to show degranulation of beta cells.[16]

Although most renal subcapsular transplants in BB rats eventually fail, in this study two rats maintained functional kidney subcapsular islets for more than 120 days, allowing us to assess the capacity of allografts in this site to induce unresponsiveness. One of these rats was grafted with Lewis skin, which was rejected after 29 days, provoking rejection of the islet graft 9 days later. The other rat received a second Lewis islet allograft, transplanted intraportally. This animal remained normoglycemic, and 60 days later was killed for histologic examination of the transplanted islets. Although noninfiltrated islets were found under the renal capsule, only a few recognizable islets remained in the liver, and these were infiltrated by mononuclear cells. Thus it appeared that the persistence of allogeneic islets in the kidney does not diminish the vigor of the immune response to subsequent allografts of donor-strain skin or extrathymic islets. The pancreata of all animals mentioned above were examined histologically and in no instance did recognizable beta cells remain.

Studies of Intrathymic Islet Allografts in Immunized Hosts

To evaluate the capacity of the thymus to act as a privileged site in presensitized hosts, four WF rats were sensitized with Lewis skin allografts. After rejection of the skin grafts, the rats were rendered diabetic with streptozotocin and 5 days later transplanted with Lewis islets into either the thymus or another privileged site (the testicle), in conjunction with 1 mL of ALS administered intraperitoneally. All animals became normoglycemic after transplantation but rejected the islets rapidly (within 10 days), indicating that the efferent arc of the immune response is intact for both intratesticular and intrathymic grafts and confirming our previous experience with allografts in other privileged sites (Table 2).[17]

The vulnerability of established intrathymic islet allografts to immunity induced by subsequent donor strain skin also was assessed. Three (two WF, one BB) rats that had maintained functional intrathymic Lewis islet allografts for 120 to 200 days were grafted with Lewis skin. Skin allograft survival in the two WF rats was minimally if at all prolonged (11, 14 days *versus* 9 to 10 days in six controls). This was also the finding in the BB recipient, in which rejection of the skin was complete in 30 days (control MST: 32 days, n = 4). Interestingly none of these animals rejected the skin grafts in an accelerated manner, indicating that they had not been sensitized by the intrathymic islets and suggesting interruption in the afferent arc of the immune response. The fate of the long-standing intrathymic islets was variable in these same animals after they were challenged with a skin allograft. In the BB rat, diabetes recurred 36 days after skin grafting (6 days after the completion of skin graft rejection). One of the WF rats became hyperglycemic 11 days after skin grafting, while the other has remained normoglycemic despite rejection of the donor-strain skin allograft.

TABLE 2. *Survival of Lewis Islet Allografts in Sensitized WF Recipients*†

Transplant Site	Days of Allograft Survival	
	Sensitized Hosts*	Unsensitized Hosts
Testicle	10, 10	50 × 2, 76, 110, >200 × 2
Thymus	5, 6	28, 33, 57, >200 × 10

† All recipients received 1 cc ALS administered intraperitoneally at time of islet transplantation.
* Islets were transplanted 5 days after rejection of a Lewis skin allograft.

In Vitro *Assays to Characterize the Unresponsive State Induced by Intrathymic Islet Allografts*

Several assays were performed to define possible differences in the immune system induced by the presence of alien antigen in the thymus. To exclude the possibility that inoculation of islets into the thymus had disrupted T-cell maturation and thus rendered the animals immunodeficient, lymph node cells from long-term (more than 120 days) BB or WF recipients of intrathymic islets were analyzed by flow cytometry (Fig. 3). Cells from unmanipulated and intrathymically grafted WF rats showed similar percentages of TCR+, CD8+, CD4+, and sIg+ subsets. While both naive and transplanted BB rats demonstrated the T-cell lymphopenia characteristic of this strain,[18] no significant phenotypic differences were noted between these two groups.

Mixed lymphocyte culture assays previously performed on LNC from WF recipients of intrathymic Lewis islets showed normal proliferative responses to donor alloantigens as compared to nontransplanted controls.[4] We did not perform these assays in BB rats because there is never a significant *in vitro* proliferative response to allogeneic stimulators.[18,19]

Adoptive Transfer Studies to Assess Possible Suppressor Cell Activity in Intrathymic Islet Recipients

Two mechanisms that could account for the unresponsiveness of long-term recipients of intrathymic allogeneic islets to subsequent extrathymic transplants are (1) deletion or inactivation within the thymus of specific T-cell populations that could otherwise be expected to destroy extrathymic grafts and (2) generation of suppressor T cells.

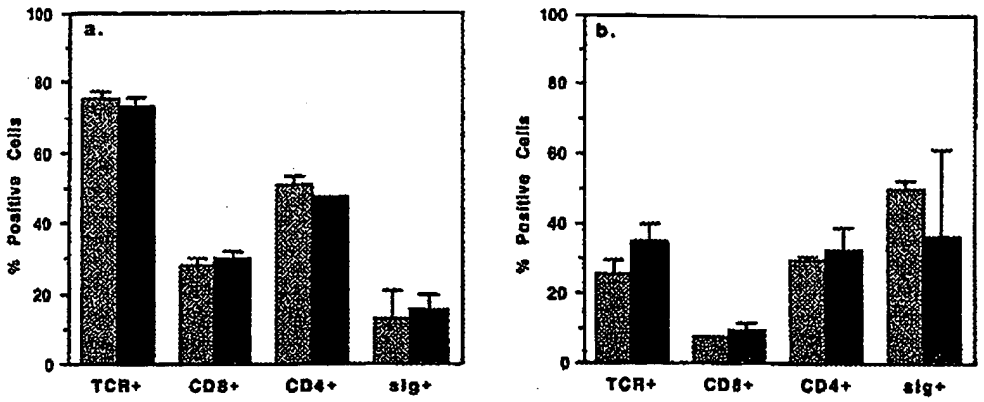

FIG. 3. Immunofluorescent analysis of lymph node cells from WF and BB rats bearing long-term (more than 120 days) intrathymic (IT) Lewis islet allografts. (a) WF controls (shaded bars); WF recipients of intrathymic islets (solid bars). (b) untransplanted spontaneously diabetic BB controls (shaded bars); BB recipients of intrathymic islets (solid bars). Results are means ± SD of three or four separate experiments.

We have already reported the results of studies to assess the first possibility (see Discussion). To evaluate the latter, we adoptively transferred 250 to 300 × 10^6 spleen cells either from nontransplanted WF controls or from WF rats harboring intrathymic Lewis islet allografts for more than 100 days to sublethally irradiated WF hosts. These animals subsequently received islets from Lewis donors beneath the renal capsule. Islet survival in rats given spleen cells from intrathymic recipients was not significantly different than that of the control group and thus provided no evidence for suppressor cells (Table 3).

Discussion

Recently we found the thymus to be another of the relatively few transplant sites that will permit successful engraftment and normal metabolic function of pancreatic islets. Even more interesting was the concomitant observation that the thymus is a previously unrecognized immunologically privileged site. Islet allografts transplanted intrathymically to nonimmunosuppressed rats with chemically induced diabetes exhibited substantially prolonged survival and, if the hosts were briefly immunosuppressed with a single dose of ALS, permanent allograft survival was achieved. Although the precise mechanisms responsible for these findings remain to be elucidated, a partial explanation is suggested by several known morphologic and physiologic characteristics of the organ. Ultrastructural studies as well as experiments using particulate dyes have demonstrated the presence of a blood–thymus barrier surrounding capillaries in the thymic cortex.[20] In addition the thymus possesses no afferent lymphatic supply.[21] These details of vascular anatomy may account for the findings of Michie et al.[22] that there is minimal recirculation of mature T lymphocytes through the thymic parenchyma. Despite the classification of the thymus as a primary lymphoid organ, it is relatively removed from the immune surveillance, that takes place in other tissues, thus explaining the sanctuary it provides for allografts.

In the present study, we sought to determine whether the thymus' immunologically privileged nature would be sufficient to sustain allografts implanted in sensitized hosts that bear an expanded population of effector T cells reactive to donor alloantigens. However, when we transplanted allogeneic islets into the thymus of recipients that had previously rejected donor strain skin grafts, the islets were destroyed in an accelerated manner, demonstrating that the intrathymic site is readily accessible at least to activated T cells. These findings correlate with reports that antigen-stimulated T lymphocytes have an enhanced capacity to recirculate through the thymic parenchyma.[23,24] That the thymus is indeed an immunologically privileged site is not contradicted by these results because it is well established that even sites of proved immunologic privilege such as the anterior chamber of the eye, the brain, and the testicle are incapable of protecting allografts from destruction in previously immunized hosts.[17] Lending further credence to the status of the thymus as a privileged site was our finding that intrathymic islet allografts did not sensitize their hosts to subsequent donor strain test skin grafts, which were rejected with normal tempo. This outcome is consistent with in-

TABLE 3. *Survival of Lewis Islets in WF Rats Inoculated with Syngeneic Spleen Cells from Intrathymic Lewis Islet Recipients*

Status of WF Spleen Cell Donor	Treatment of Recipient*	Days of Islet Allograft Survival
Intrathymic Lewis islets†	450R	8, 9, 12
Naive	450R	12, 14, 16

* Rats were irradiated on day −2 and received 250 to 300 × 10^6 spleen cells on day −1 relative to islet transplantation.

† Animals had retained intrathymic Lewis islet allografts for more than 100 days.

terruption of the afferent arc of the immune response, which is also a characteristic of most other privileged sites.

While the prolonged survival of intrathymic islets in naive hosts may be explained by the immunologically privileged status of the thymus, the finding that recipients bearing established intrathymic grafts are unable to reject subsequent donor strain islets suggests that mechanisms that alter systemic alloimmune responses are also contributory. We have previously demonstrated that frequencies of cytotoxic T-cell precursors specific for donor alloantigens are reduced in intrathymic islet-bearing hosts.[4] Thus one such mechanism may involve deletion or functional inactivation of alloreactive clones by the foreign cells within the thymus. Nevertheless the possibility remains that additional mechanisms such as generation of suppressor or anti-idiotypic T cells may contribute to the acceptance of extrathymic grafts. In fact suppressor cell-mediated unresponsiveness has been shown to develop in several models of long-term graft survival, particularly those in which acceptance of the allograft is achieved by administration of potent immunosuppressive agents.[25,26] Our finding that islet allograft survival in secondary hosts was not prolonged by transfer of lymphoid cells from intrathymic islet-bearing recipients argues against the generation of regulatory cell populations as the basis for the survival of intrathymic islets and further supports the conclusion that the unresponsive state to extrathymic grafts is mediated primarily by deletion or inactivation of specific T-cell clones within the thymus. That skin allograft survival was not prolonged by the intrathymic islets was somewhat disappointing because it indicates that the degree of unresponsiveness we have induced by this method is not comparable to that which is caused by neonatal inoculation of donor-strain bone marrow, and thus should probably not be referred to as "tolerance."[27]

A central focus of the present study was to test the capacity of the thymus to protect islets transplanted to spontaneously diabetic BB rats. It is important to remember that these transplants were done in hosts that had already destroyed their native pancreatic islets. In this sense they were presensitized to islet tissue and presumably had an expanded population of effector cells reactive to putative autoantigens in the transplanted islet tissue.[28] The barrier of autoimmune damage to islets may, in fact, be at least as important to the success of islet transplantation as rejection because in both animals and humans it can result, by itself, in islet failure.[29] Viewing the experiment in this context, it is rather surprising that the outcome of intrathymic islet transplantation was so good. In fact in none of the 11 acutely diabetic BB rats transplanted with Lewis islets was there ever an indication from blood glucose values or histology that even mild rejection or autoimmune insulitis occurred. In contrast all intraportally transplanted islets were rapidly and completely destroyed. In addition to these controls, an extensive collective experience exists from our previous work and that of others concerning the fate of islets transplanted to other extrathymic sites in BB rats (reviewed in Markmann et al.[29]). We and others have noted that transplanted islets are destroyed by BB rats even when the contribution of allogeneic rejection is eliminated by pretransplant *in vitro* culture or by the induction of neonatal tolerance. Thus in no previous reports, despite the fact that BB rats are relatively immunodeficient, could failure from rejection or autoimmune damage be consistently prevented unless islets were subjected to pretransplant culture or unless heavy or continuous immunosuppression was used. For example, when Selawry et al.[30,31] transplanted islets to a privileged site (the testicle) of BB rats, they found it necessary to use either pretransplant tissue culture or ALS (or both) to obtain prolonged survival. Neither pretransplant culture or immunosuppression was used at any stage in the experiments in BB rats reported here.

The possibility that autoimmune destruction of islets is an MHC-restricted process raises another interesting question: Will MHC-compatible islets fare worse than MHC-incompatible ones in BB rats because MHC restriction might protect the latter from autoimmune destruction?[12] Regardless of this consideration, we found the intrathymic site to be protective because both uncultured WF and Lewis islets implanted there uniformly survived while those transplanted to the renal subcapsule or liver did not.

While the prolonged survival of intrathymic islets in BB hosts must, at least in part, be due to the immunologically privileged status of the thymus, the finding that recipients bearing established intrathymic grafts fail to destroy subsequent extrathymic islets either by rejection or autoimmunity argues that additional mechanisms that alter systemic immune responses are also involved. Although the evidence for this presented here in BB rats is good, it is not quite as firm as that in our previous report on the weakening of the alloimmune response we caused in chemically diabetic rats. In three of the four BB rats that were retransplanted with intraportal islets after prolonged residence of an intrathymic graft, the persistence of normoglycemia after removal of the thymus demonstrated that the intraportal graft was not damaged by either rejection or autoimmunity, a conclusion later confirmed histologically. In the fourth rat, however, removal of the thymus did cause hyperglycemia. Examination of the liver of this rat revealed many islets that stained only weakly for insulin granules. No mononuclear infiltrate was noted. This finding is compatible with either of the following explanations: (1) the number of islets transplanted intra-

portally was insufficient to maintain normoglycemia and the remaining beta cells degranulated; (2) there was specific autoimmune destruction of islet beta cells while the nonbeta cell populations were protected from rejection by the influence of the intrathymic allograft.

Somewhat surprisingly and in contrast to our earlier experiments in streptozotocin-diabetic WF rats, it was not necessary in BB rats to use any immunosuppression to achieve permanent survival of intrathymic islet allografts or to induce unresponsiveness to subsequent extrathymic ones. A possible explanation for this is that the profound T-cell lymphopenia characteristic of BB rats allowed intrathymic islets to survive long enough to effect unresponsiveness. However, despite the immunodeficient status of BB rats, the eventual destruction of the majority of intraportal and renal subcapsular islet allografts demonstrates that these animals retain the capacity to respond to foreign tissue grafts and further underscores the superior nature of the thymus as a transplantation site.

We previously postulated that the donor-specific unresponsive state induced by intrathymic islet transplantation develops as T lymphocytes maturing in the thymus become tolerant to the antigens present within this specialized microenvironment. In animals that are fully immune competent, this effect may require transient depletion of mature peripheral T cells to prevent destruction of the intrathymic graft. However, if prolonged allograft survival in the thymus is achieved on some basis other than immunosuppression, gradual physiologic replacement of the peripheral T-cell repertoire by newly matured T cells may produce similar results. Although evidence exists that a sizeable percentage of peripheral T cells are relatively long lived,[32] the prolonged interval between intrathymic grafting and subsequent intraportal retransplantation (more than 120 days) used in our studies of BB rats may have been sufficient for extensive repopulation of the periphery with "tolerant" lymphocytes. On the other hand, it is possible that, in contrast to our findings using ALS-treated rats, the presence of intrathymic grafts in nonimmunosuppressed hosts can lead to development of regulatory cell populations that inhibit immune responsiveness to donor alloantigens. We are currently addressing this possibility.

This report provides further evidence that the success of intrathymic transplantation of islet allografts depends on the immunologically privileged status of the thymus as well as on systemic alterations in the immune system that this approach induces. Furthermore our demonstration that intrathymic islet transplantation can restore permanent euglycemia in spontaneously diabetic BB rats indicates that the thymus can protect islets from both allogeneic rejection and destruction by recurrent anti-beta-cell autoimmunity. The applicability of this approach to the treatment of human type I (insulin-dependent) diabetes mellitus remains to be determined.

Acknowledgments

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Discussion

DR. ANTHONY P. MONACO (Boston, Massachusetts): It is a pleasure for me to discuss Dr. Naji and Dr. Barker and their group's paper. I have had the privilege of also reading it last night. This is a provocative and outstanding and highly intelligent work. It is a continuum of their extraordinary work over the years, and they never disappoint us. This work is another example of their extraordinary scientific ability and intellectuality.

In their paper today, they have demonstrated that the thymus seems to have the characteristics of a privileged site; that is, that islets placed in the thymus seem not to be rejected. In their manuscript, which I had the opportunity to read, if that experiment is done in a sensitized animal, that is, sensitized to the histocompatibility antigens of the islets placed in the thymus, the experiment does not work, and those islets are rejected.

This is characteristic of the classic privileged site phenomenon. What is different is that residence in the thymus seems to confirm some extrathymic message in the form of extrathymic tolerance. This tolerance, however, also is kind of unique. At least we do not have all the information to determine whether it is typical tolerance of the Billingham-Brent-Medalar type.

First of all when islets were transplanted later extrathymically, they were not rejected as long as islets were in the thymus. We would like to know whether this tolerance extends to similar tissues of the same histocompatibility type, such as skin or liver, or some other tissue, or whether it is unique just for the thymus.

Furthermore this phenomenon still probably needs some extra thymic immunologic ablation because, when it is done not in the BB rat, they have had to use antilymphocyte serum, and, as Drs. Naji and Barker point out in their paper for me, this BB rat has a certain amount of immunodeficiency.

So I have three questions. First have you done any experiments to see whether the tolerance extends to nonislet tissues? Secondly, and most important, if this is an exclusion of the T-cell r
ertoire of the MHC of the islet, then it should be able to be accomplished by putting any tissue of the same MHC in that thymus, like lymphocytes, splenocytes, etc.

And then thirdly, does the type of challenge that one does peripherally affect the tolerance that you get? That is if you have more of an immunogenic challenge, is the tolerance broken?

It is a pleasure for me to discuss this extraordinary paper.

DR. FRANK THOMAS (Greenville, North Carolina): Our group has also been interested in this area, and the mechanism by which a striking tolerance to islets is achieved, the subject addressed by these studies. We agree that suppressor cells are unlikely to explain this phenomenon.

Cells reactive to donor islets developed by both positive and negative selection in the thymus, an organ described as the place T cells go for their college education. As first described by Miller, Bevan, and others, a number of cells can act within the thymus to execute what has been called a veto phenomenon, effectively vetoing the antidonor activity of a developing thymic cell.

Although commonly involving T cells, the veto mechanism can involve macrophage and dendritic cells, cells acting in concert, some think even with somatic donor cells, which of course would include donor islets. Our group has identified the veto phenomenon in a model of adult tolerance we have studied, and found it to be a quite potent mechanism.

The key feature of the veto mechanism is the ability to signal developing T cells within the thymus to abrogate their programmed cytotoxicity to the donor or veto antigens. Many of the features described by Dr. Naji's group, including clonal deletion or energy as shown by MLC and CTL precursor studies, fit the veto phenomenon to a T.

The uniqueness of the thymus among body organs in generating this reaction is also quite in keeping with the veto phenomenon that occurs largely in the thymus by classical thinking.

Have you entertained the veto hypothesis to explain intrathymic-induced tolerance? There are definitive ways by which this hypothesis could be studied beyond phenotypic analysis of total thymus T-cell maturation and MLC studies performed. I wonder if you have pursued any of these studies to date?

DR. MARK A. HARDY (New York, New York): This is a most exciting and important study, and I would like to join all the others in complimenting the authors on the thorough and complete study, which I had the privilege to review last night in manuscript form.

This approach of intrathymic injection of antigen to induce immunologic unresponsiveness has really a much broader implication in transplantation than just for islet transplantation, as our own work now has shown that the use of intrathymic UVB-irradiated splenocytes permits indefinite cardiac allograft survival.

Another recent paper in *Lancet*, using isolated glomeruli injected intrathymically in a rat model, allowed subsequent donor-type kidney transplant survival in these rats.

It is therefore conceivable that the use of the approach described by the authors may permit specific clinical manipulation of the host to allow prolonged survival of allografts other than islets and thus avoid or at least reduce the toxic side effects of immunosuppression. To be clinically practical, however, the interval between the intrathymic injection and the subsequent grafting must be very short, of the order of a day or two, to adhere to the way that we preserve organs today.

Therefore I would like to ask the authors whether they have tried to shorten the interval between injection and grafting. The interval that they have used is about 100 days. In our experiments, we have used an interval of a week. This question has to be addressed very carefully.

Have they tried this approach in larger animals, as we are looking for a clinical application?

Theoretically what do they think is the role of the donor antigen-presenting cells *versus* the intrathymic antigen-presenting cells in the development of this type of nonself- and self-recognition?

What are the kinetics of the allograft unresponsiveness in this model? Specifically have they removed the thymus at any point before subsequent grafting to see whether the beneficial effect continues? Have they fulfilled in that sense Koch's postulate?

And finally I would like to ask, do they think that the organ-specific antigens play a role in induction of specific organ unresponsiveness?

I enjoyed reviewing this paper. I think it is one of the most exciting and important contributions, at least in the last year, to the field of transplantation. And I look forward to much more work on this subject in the future.

I want to thank the society for the privilege of the floor.

DR. JOSEPH E. MURRAY (Boston, Massachusetts): I join the others in acknowledging this superb experimental model. I have always been intrigued by the lag time in many aspects of scientific progress. In this instance there is a 19-year interval from the original description by Raviola and Karnovsky of a blood–thymic barrier in the thymic cortical capillaries to its use in experimental models. As soon as I return home, I will look up that original paper to find out just why they were pursuing that particular phase of thymic anatomy.

This time lag is reminiscent of the lag between Lillie's 1916 observation of the placental intermingling of blood in freemartin cattle and the 1945 report of Ray Owens on the immunologic consequences of this anatomic anomaly. Owens' report led directly to the Billingham, Brent, Medawar paper in *Nature* in 1953 on "Actively Acquired Immunological Tolerance in Mice," a foundation piece in transplantation biology, for which Medawar received a Nobel Prize.

Raviola and Karnovsky apparently traced the lymphatics in the thymus and found absence of the efferent component. Like Dr. Monaco I am intuitively attracted to all possibilities in transplantation that might eliminate the need for immunosuppressive drugs. There are hints that naturally occurring immunosuppressive agents may exist. Dr. John Mannick and James Mobray published on such a serum factor years ago.

Sir Roy Calne noted 20 years ago that the pig liver seemed to possess a natural immune tolerance. Currently his group in Cambridge, England have reported in rats that the serum from successfully allografted liver recipients is protective when tranfused into untreated liver recipients. This observation reminds me of Nate Kaliss's enhancing antibody, which protected tumors from being rejected. Recently a Canadian group has reported that human intestinal allografts seem to survive and function better in the presence of a liver allograft. These observations may be clues for the future.

You state in your manuscript that your animals are not tolerant in the Billingham, Brent, Medawar sense. Billingham, Brent, and Medawar used to wonder whether they were producing tolerant cells or tolerant animals. How would you answer that question today on the basis of your current experience?

Finally I was surprised that the renal capsule was partially a privileged site. I enjoyed this paper very much not only for its clinical but also for its biologic implications. The model applications may well become a classic.

DR. JAMES C. THOMPSON (Galveston, Texas): I thought I might break into this Transplantation Mafia here. My ignorance of the thymus amounts to a national resource, but I would like to ask the authors about duration of thymus activity. We know that the thymus gradually stops working after a while; and at what age does it lose this ability to effect this kind of magic transmutation?

And if that has been studied, is there any way to prolong this immune protective function of the thymus, stick in a new one, or anything like that?

DR. JOHN S. NAJARIAN (Minneapolis, Minnesota): The authors have described an intriguing possibility regarding the formation of some form of immunologic tolerance. They have demonstrated that this is an organ-specific phenomenon, because skin graft from a donor of the same strain is rejected.

I did not get a chance to read the manuscript, but I am intrigued by the immunologic question it raises. That is if you implant islets into the thymus, remove the thymus containing the islets, and then challenge the host with islets from a rat of the same strain, do the second islets survive? In other words do you have cellular tolerance to that specific organ? If so that is the first report I have seen of immunologic tolerance in which persistent antigen has not been present.

And so this is my final question: Have you done this experiment and does it work? When you remove the thymus containing the islets, will a second transplant of islets from the same strain survive?

DR. ALI NAJI: Dr. Murray, the origin for the idea of intrathymic cellular transplantation is the classic paper of Billingham, Brent, and Medawar in *Nature* (172:603, 1953). In this landmark report, which I have reread many times, the authors described the concept and the feasibility of induction of neonatal tolerance in mice inoculated with cells of lymphoid and nonlymphoid tissues such as testes and kidneys. Curiously the total number of animals rendered tolerant was actually small. One wonders if the inefficiency of the tolerance-inducing inoculum was due to the proportion of lymphoid and nonlymphoid cells or to the technical difficulty of targeting the cells to the fetus. It is well known that neonatal inoculation of cells of lymphohematopoietic origin routinely produces a high degree of tolerance. We reasoned that the failure of nonlymphoid cells to induce tolerance may have been related to inability of such cells to "home" to the thymus, whereas cells of lymphohematopoietic origin would be expected to migrate to the thymic microenvironment. Thus we initiated experiments to assess whether cells of nonlymphoid origin if implanted in the microenvironment of the thymus would be capable of inducing tolerance.

We have found that the acquisition of peripheral immune unresponsiveness after intrathymic islet inoculation permits indefinite survival of extrathymic islets, but only slight prolongation of skin allografts. It appears that the thymus behaves as a classic immunologically privileged site and is subject to the usual biologic characteristics of such sites, in that prior sensitization of the host with skin allografts precludes prolonged survival of intrathymic islets.

The question of why occasional islet allografts survive permanently in the renal subcapsule without a prior intrathymic implant is intriguing. The duration of these islet survival was variable, however, and on long-term observation they were all rejected. In contrast virtually all intrathymic islets were protected against rejection and produce a remarkably superior degree of blood glucose homeostasis than intraportal or subrenal capsule islet allografts.

Dr. Najarian, you asked whether the peripheral unresponsiveness could be maintained after the removal of the thymus bearing the alloantigen. We have not carried out extrathymic islet transplants after removal of the thymus, but to some extent our work in the BB rats addresses your question. In our experiments the spontaneously diabetic BB rats were transplanted with intrathymic islets and approximately 100 days later were challenged with an extrathymic (intraportal) allogeneic islets. The BB recipients harboring both intrathymic and intrahepatic islet allografts were observed for an additional 2 months before removal of the thymus bearing the islet allografts. In thymectomized hosts observation for an additional month showed no destruction of the extrathymic islets or recurrence of diabetes. Therefore it appears that the continued presence of the thymus bearing allogeneic islets is not necessary for maintenance of the peripheral unresponsiveness.

Dr. Thompson asked whether the involution of the thymus with age influences the survival of the intrathymic islets. We were initially concerned that with the progressive involution of the thymus we might observe destruction of the islets and recurrence of diabetes. The recipients in our studies were all adults, however, and transplants were carried out at 10 to 12 weeks of age. We have now followed other intrathymic islet recipients for more than 2 years, a period close to the life span of the rat, without any recurrent diabetes.

Dr. Hardy, we are delighted that Dr. Remuzzi and his colleagues from Italy (*Lancet* 337:750, 1991) have confirmed our work by their recent report of prolonged survival of renal allografts after intrathymic glomerular transplantation.

The question of the requirements of the donor *versus* recipient antigen-presenting cells is an important one. We have addressed this issue by intrathymic transplantation of *in vitro* cultured islets to delete intra-islet APCs. This strategy permits us to dissect the contribution of islet endocrine cells (expressing class I major histocompatibility complex [MHC] antigens) or the intra-islet antigen-presenting cells (expressing both class I and class II MHC antigens) toward induction of peripheral unresponsiveness. Our preliminary data indicate that APC-depleted intrathymic allografts survive indefinitely. Interestingly APC depletion obviates the need for administration of a single dose of antilymphocyte serum to reduce the peripheral mature T lymphocytes. We have not assessed the

precursor frequencies of the cytologic T lymphocyte (CTL) or helper T cells to assess the tolerogenic potential of class I or class II MHC antigen-bearing cells, however.

Dr. Thomas's remarks were related to the veto phenomenon as a mechanism for the induction of peripheral unresponsiveness. The veto phenomenon as described by Fink and Bevan assumes an interaction between two lymphoid cell populations. I am not aware that nonlymphoid cells, such as islet endocrine cells, can participate in the veto network phenomenon. We have assumed that the peripheral unresponsiveness is due to the persistence of the alloantigen-bearing parenchymal cells (islet endocrine cells) in the thymic microenvironment and their influence on maturing thymocytes. There is evidence that maturing thymocytes are more susceptible to tolerance-inducing signals and moreover that "inappropriate" presentation of antigen by nonlymphoid cells induce a state of anergy in T cells.

Dr. Monaco asked about the abolition of the tolerance by reconstitution of the hosts with syngeneic cells. As you know this constitutes a classic method to abolish neonatal tolerance, and we have begun to assess the impact of the syngeneic lymphoid cell reconstitution on abolition of tolerance after intrathymic islet transplantation. We have carried out further studies on the efficacy of intrathymic transplantation of cells of lymphohematopoietic origin to induce peripheral unresponsiveness. Our preliminary results are encouraging, in that intrathymic inoculation of lymphohematopoietic cells possessing tolerogenic potential appears to induce peripheral unresponsiveness toward other tissue and vascularized organ allografts.

LATTA.002C4 PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Latta, Paul P.
Appl. No.	:	10/823,263
Filed	:	April 13, 2004
For	:	A METHOD OF TREATMENT OF DIABETES THROUGH INDUCTION OF IMMUNOLOGICAL TOLERANCE
Examiner	:	Belyavski, Michail A.
Group Art Unit	:	1644

DECLARATION OF DAVID SCHARP, M.D.

UNDER 37 C.F.R §1.132

Commissioner for Patents

P.O. Box 1450

Alexandria, VA 22313-1450

Dear Sir:

1. I, David Scharp, M.D., am Executive Vice President and Chief Medical Officer of Novocell, Inc. At Novocell I am actively engaged in research related to development of treatments for diabetes using encapsulated insulin-producing cells.

2. I have extensive experience in the field of the claimed invention as indicated in the attached Curriculum Vitae provided herewith as Exhibit A.

3. Working with the sole inventor of the present application, Paul P. Latta, and others, I carried out experiments to evaluate the efficacy of treating diabetes through induction of immunological tolerance.

4. The currently available approach to treating diabetes, which does not require repeated administration of insulin, is transplantation of insulin-producing cells from a donor to the diabetic patient. However, the problem with this approach is that such transplants do not survive the attack of the host immune system unless the patients are under continuous, life-long immunosuppression.

5. The approach of the present invention is totally different in that we are implanting a very small fraction of a therapeutic dose of encapsulated islet allografts prior to implantation of the fully therapeutic dose in order to tolerize the immune system to the foreign antigens shed by these encapsulated allografts made impervious to the assault by the host immune system. The fraction of encapsulated islets in the tolerizing dose is so small it would have no effect on controlling blood glucose by itself. However, the continuous release of donor antigens from this small fraction of encapsulated islets not destroyed by the host, alters the immune response in a way that the host no longer considers the implanted islets to be foreign. The tolerization therefore, protects the later implanted non-encapsulated islet allografts from being rejected by the host, allowing them to take permanent hold in the host body and produce the fully therapeutic amount of insulin as needed to treat diabetes.

6. In our original experiments, described in the Specification of the above-identified application, we induced diabetes in mice by intravenous injection of streptozotocin. Induction of diabetes by streptozotocin injection is a well-known procedure which destroys pancreatic insulin-producing cells. We first implanted into these mice a small, sub-therapeutic dose of encapsulated insulin-producing cells. Two to three weeks later the same mice received an additional, large (therapeutic) unencapsulated implant (2,000-3,000 cell aggregates, 1000 cells per aggregate) of insulin-producing cells. We showed that the encapsulated insulin-producing cells given as a small mass of 100 cell aggregates (1,000 cells per aggregate) permits this second unencapsulated implant to survive as shown by normalized blood glucose levels in the treated mice.

7. We then turned to another animal model of diabetes, non-obese diabetic (NOD) mice, and proved that the method works in this model as well.

8. There are two available animal models for studying Type I diabetes, the BB rat and the NOD mouse. The BB rat, while developing diabetes, has a multitude of immunologic disorders that makes it more of a model for immune deficiency than diabetes. The BB rat is no longer considered an acceptable model for studying human autoimmune diabetes.

9. The NOD mouse is therefore, the only animal model for autoimmune, Type I diabetes that it is predictive of human disease. The lymphocytes of the NOD mouse spontaneously begin attacking its insulin-producing pancreatic beta cells soon after birth. Looking at the histology of the pancreas of these mice during the autoimmune process, one finds early that most islets are infiltrated with immune cells that are destroying islets. As the process continues towards the diabetic phase, almost all of the beta cells are destroyed leaving smaller than normal islets with residual inflammation that continues to destroy the new islets that are stimulated to develop due to the failing islet mass. This ongoing destruction of the insulin-producing pancreatic beta cells continues and progresses for 15 to 32 weeks until a sufficient number of beta cells are destroyed to cause the clinical onset of Type I diabetes in NOD mice. Examining living NOD mice prior to development of clinical diabetes, including monitoring their blood glucose levels, one would have no clue that this autoimmune process is actively destroying their pancreatic beta cells. Yet, if one examines their blood for anti-islet protein antibodies, one can clearly identify those animals that will eventually lose blood glucose control and develop clinical diabetes. This situation is identical for human Type I diabetes in that patients at high risk for developing Type I diabetes are tested for the presence of specific auto-antibodies. The number and titers of these specific antibodies can predict with >90% certainty which of these patients with ongoing autoimmune destruction of their beta cells will actually develop clinical Type I diabetes within 5 years.

10. We encapsulated islets from mouse strain C57Bl/6 by polyethylene glycol conformal coating as described in USP 5,529,914. This patent was incorporated by reference into the specification of the application captioned above. We then implanted these conformally-coated islets by intraperitoneal injection into NOD mice. The experiment had two variables under study

– time of implantation of the encapsulated islets (4, 8, and 12 weeks) and number of islets implanted (50, 100 and 150 islet equivalents, IEQ).

11. **Exhibit 1** shows the data for all animals grouped by time of implantation. Implantation at 4 weeks showed the best results with diabetes being prevented in 60% of the treated animals, as compared to the control animals with none having diabetes prevented.

12. **Exhibit 2** shows the data for all animals grouped by number of islets implanted. A dose of 50 IEQ produced the best results with diabetes being prevented in 60% of the treated animals. All the control animals developed diabetes.

13. **Exhibit 3** demonstrates that in the control recipients (NOD mice not implanted with encapsulated islet cells), the autoimmune destruction of the pancreatic islets is very complete with small shrunken islets remaining with continuing evidence of lymphocytes destroying any new islets that are formed.

14. **Exhibits 4 & 5** demonstrate that in those recipients that were prevented from developing diabetes after implanting the small quantity of encapsulated islets, very large islets (many times their normal size) are present, without evidence of host lymphocyte destruction. This means that the normal process in the mouse to replace lost islets has been successful to the point of preventing diabetes from destroying all of the newly formed islet cells.

15. Therefore, we have shown that: a) a small, sub-therapeutic tolerizing dose of encapsulated insulin-producing cells prevents the host immune system from attacking a later-implanted un-encapsulated therapeutic dose of insulin-producing cells; and b) a small, sub-therapeutic tolerizing dose of encapsulated insulin-producing cells prevents the host immune system from attacking the host insulin-producing cells in the pancreas.

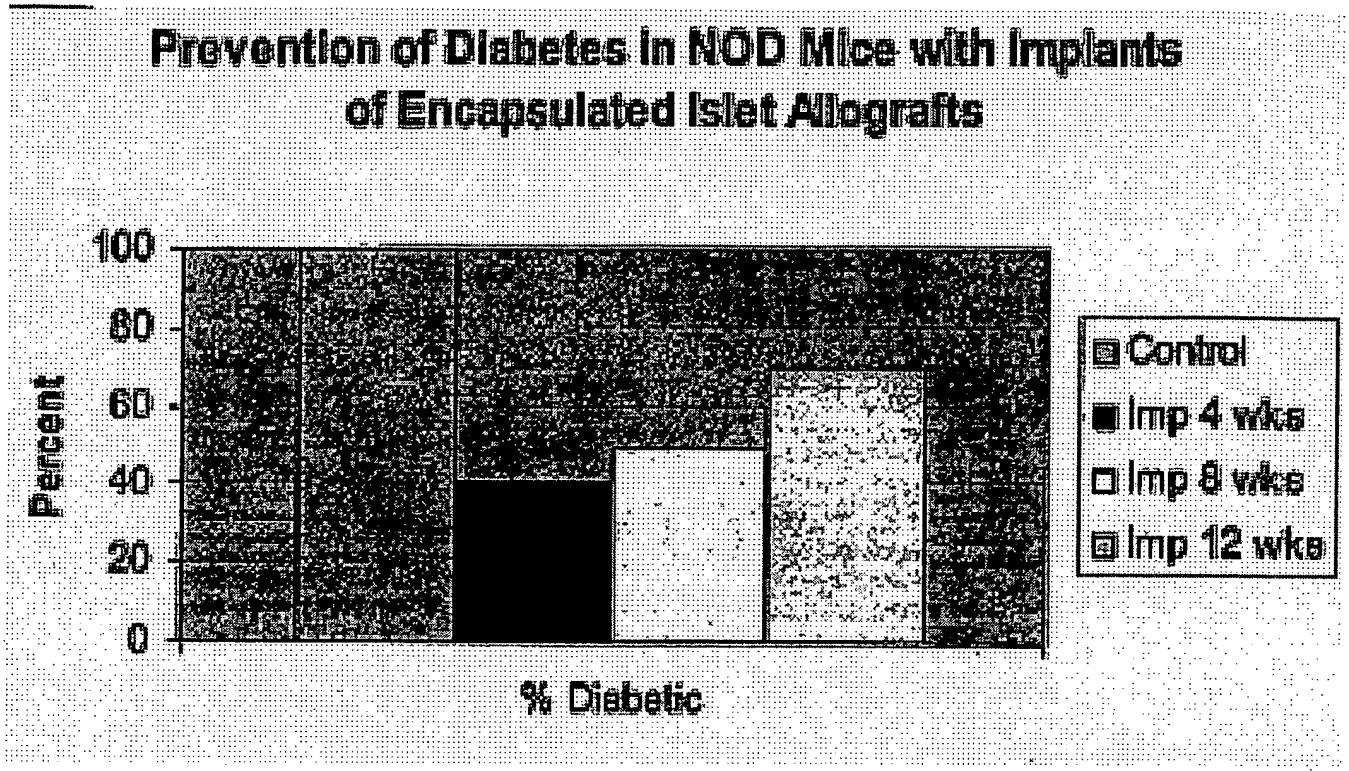
16. Combined, these experiments prove that the claimed method of treating diabetes by tolerizing the host immune system prior to implanting the fully therapeutic dose of the insulin-producing cells works to permit the host to receive the fully therapeutic dose without rejection. This process works without the need of continuous, life-time immunosuppression of the host.

17. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these

statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or patent issuing therefrom.

Dated: May 4, 2005 By: _____

David Scharp


Prevention of Diabetes in NOD Mice with Implants of Encapsulated Islet Allografts
Percent
100
80
60
40
20
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% Diabetic
Control
Imp 4 wks
Imp 8 wks
Imp 12 wks

Exhibit 1

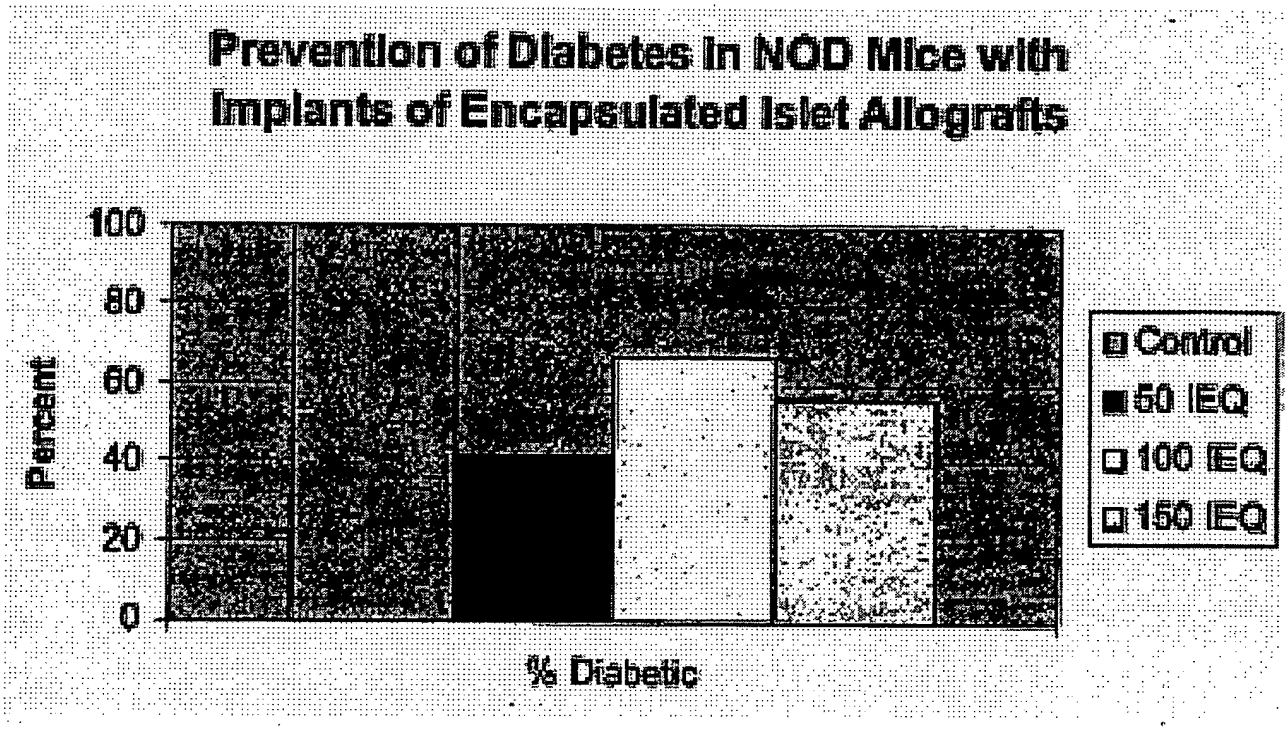
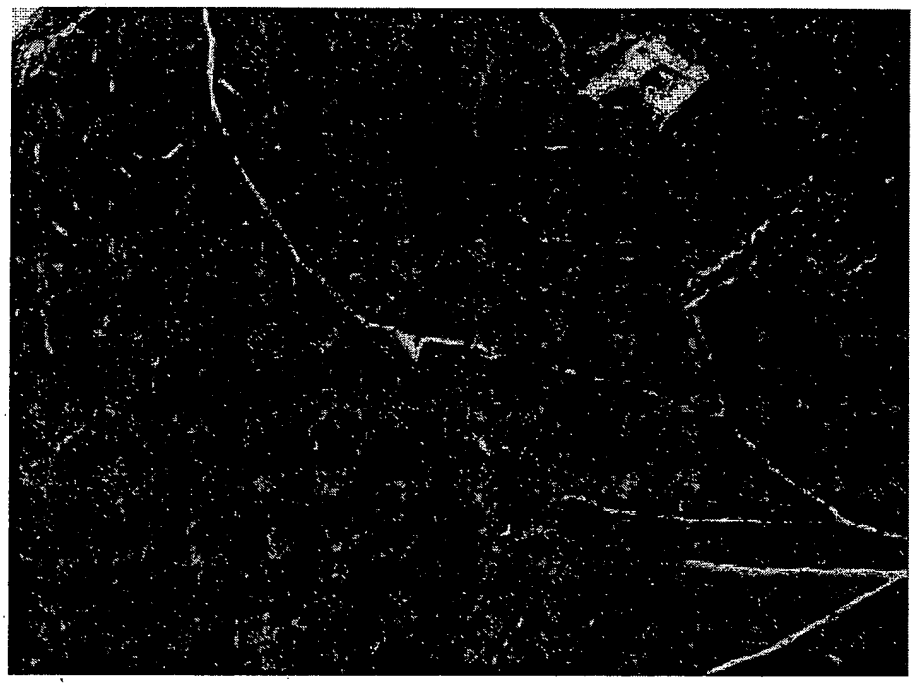

Prevention of Diabetes in NOD Mice with Implants of Encapsulated Islet Allografts
100
80
60
40
20
0
Percent
% Diabetic
Control
50 IEQ
100 IEQ
150 IEQ

Exhibit 2



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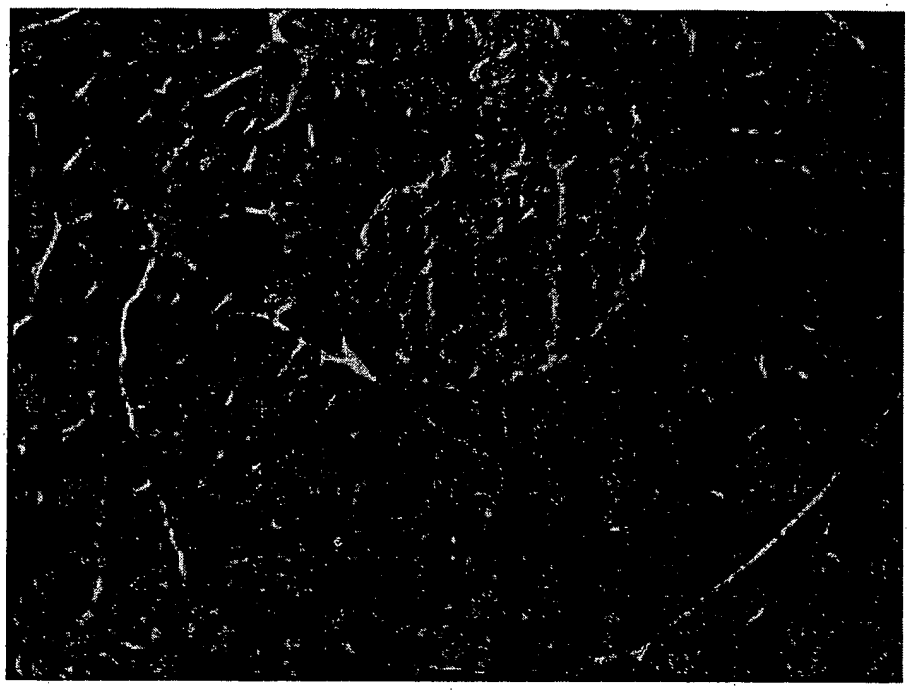

Exhibit 4

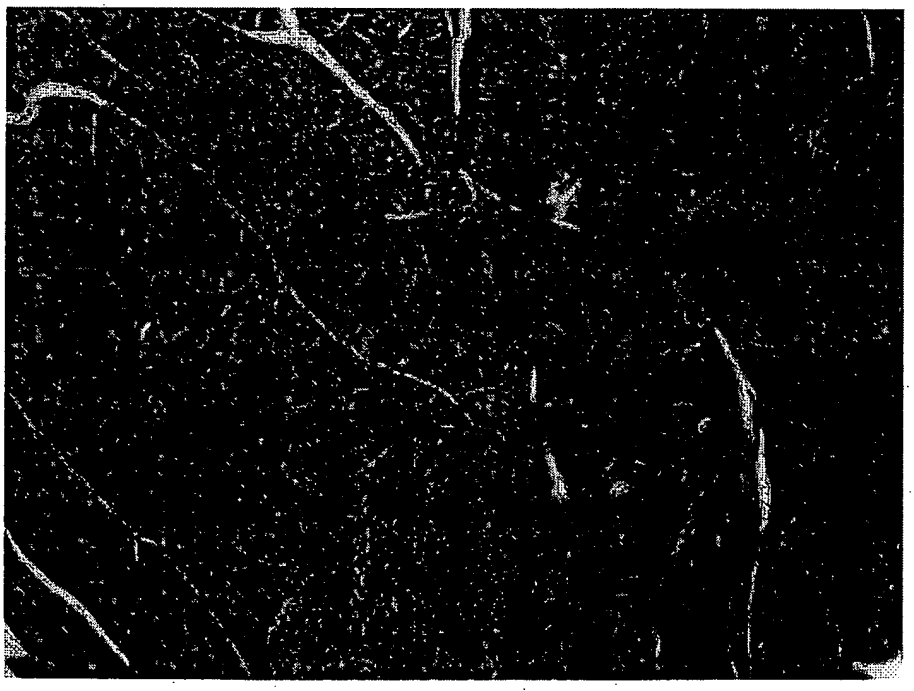

Exhibit 5

Exhibit A

CURRICULUM VITAE

David William Scharp, M.D.

Born: July 5, 1945
Washington, Illinois

Social Security Number: 327-36-7524

Marital Status: Wife: Jeanette

Children: Kevin Scharp
Daniel Scharp
Anna Scharp
David Bondurant
Melissa Bondurant

Pre-Medical Education: University of Missouri
Columbia, Missouri
1963-1966

Medical Education: Washington University School of Medicine
St. Louis, Missouri
1966-1970

Graduate Hospital Experience:

Intern in Surgery
7/1/70-6/30/71
Barnes Hospital/Washington University

Surgical Resident
7/1/71-6/30/72 & 7/1/74-6/30/76
Barnes Hospital/Washington University

Surgical Research Fellow
7/1/72-6/30/74
Washington University - Department of Surgery

Academic Positions:

Assistant Professor of Surgery
1976-1983
Washington University School of Medicine

Associate Professor of Surgery
1983-1991
Washington University School of Medicine

Professor of Surgery
1991-President
Washington University School of Medicine

Leave of Absence
1/1/94-7/1/95

Commercial Positions:

Novocell, Inc.
Chief Scientific Officer

Neocrin Company
Chief Scientific Officer 1/1/94-7/31/99
Executive Vice President of Medical Affairs 1/1/94-7/31/99
Executive Vice President for Research 1/1/94-11/1/95
Executive Vice President, Research and Development 11/1/95-President

McDonnell Douglas Corporation 1984-1987
Contractual Research Investigator
Electrophoretic Separation of Islet Cells

Cytotherapeutics, Inc. 1989-1993
Founding Scientist
Scientific Advisory Board Member
Contractual Research Investigator

Patents:

"Islet Isolation Process"
DE191613T – 1987
DE3650662D – 1998
EP0191613 – 1986
EP0191613 – 1989
EP0191613 – 1997
JP61183226 – 1986
US4868121 – 1989
US5322790 – 1994

"Method to Isolate Clusters of Cell Sub-Types from Organs"
AU1934988 – 1989
CA1340406 – 1999
EP0382727 – 1990
EP0382727 – 1991
JP2504222T – 1990
US5079160 – 1992
WO8809667 – 1988

"Implantable Biocompatible Immunoisolatory Vehicle for Delivery of Selected Therapeutic Products"
AT156344T – 1997
AU666118 – 1996
AU682796 – 1997
AU2004192 – 1992
AU3902095 – 1996
CA2109085 – 1992
CA2109085 – 2003
DE69221484D – 1997
DE69221484T – 1998
DK585368T – 1998
EP0585368 – 1994
EP0585368 – 1994
EP0585368 – 1997
ES2107537T – 1997
FI934545 – 1993
FI934545D – 1993
GR3025301T – 1998
HK1001832 – 1998
JP6507412T – 1994
NO308198B – 2000
NO933842 – 1993
SG47470 – 1998

US5798113 – 1998
US5800828 – 1998
US5871767 – 1999
US6083523 – 2000
US6322804 – 2001
US2002150603 – 2002
WO9219195 – 1992

"Methods for Coextruding Immunoisolatory Implantable Vehicles with a Biocompatible Jacket and a Biocompatible Matrix Core"
US5800829 – 1998

"Methods for Treating Diabetes by Delivering Insulin from Biocompatible Cell Containing Devices"

US5869077 – 1999

"Methods for Making Immunoisolatory Implantable Vehicles with a Biocompatible Jacket and a Biocompatible Matrix Core"
US5834001 – 1998
US5874099 –1999

"Use of Pouch for Implantation of Living Cells"
AU4788993 – 1994
CA2140905 – 1994
EP0655910 – 1995
EP0655910 – 1996
JP8500033T – 1996
US5916554 – 1999
WO9403154 – 1994

Hospital Appointments:

Assistant Surgeon 1976-1983
Associate Surgeon - 1983-Present
Barnes Hospital, St. Louis, Missouri

Attending Surgeon 1982-1985
Consulting Surgeon 1976-1982
Associate Chief of Surgery 12/86-9/90
Veterans Administration Medical Center, St. Louis, Missouri

Consulting Surgeon 1985-7/95
Acting Chief of Surgery 3/86-12/86
St. Louis Regional Hospital, St. Louis, Missouri

Consulting Surgeon 1976-1985
St. Louis Children's Hospital, St. Louis, Missouri

Consulting Surgeon 1976-1985
Chief of Surgery 1981-1982
St. Louis County Hospital, St. Louis, Missouri

Attending Surgeon 1976-1982
St. Louis City Hospital, St. Louis, Missouri

Licensure: Missouri 1970

Certification: American Board of Surgery 1979
Fellow American College of Surgery 1982
Recertification: American Board of Surgery 1989

Medical Societies:

American College of Surgeons
American Diabetes Association
American Federation of Clinical Research
American Pancreatic Association
American Society for Artificial Internal Organs
American Surgical Association
Association for Academic Surgery
Society of University Surgeons
Tissue Culture Association
American Society of Transplant Surgeons
The Cell Transplantation Society
United Network of Organ Sharing - Region 8
International Pancreas & Islet Transplantation Association

Honors and Awards:

St. Louis Surgical Society Award for Research
Recipient 1973 & 1974
NIH Research Career Development Award
Recipient 1977-1982
NIH NAIMMDD Site Visit Teams
Member 1980-1995

NIH Surgery, Anesthesiology, and Trauma Study Section
Member 1985-1989
Reserve Member Status 1989-1995
World Journal of Surgery
Guest Editor - Islet Transplantation Symposium 1984
"Separation of Islet Cells in Microgravity by Continuous-Flow Electrophoresis", NASA - McDonnell Douglas Astronautics Corp. - STS-8, Space Shuttle, "Challenger", Experiment 1984

Editorial Reviewer:
Diabetes, Surgery, Journal Clinical Investigation
Grant Reviewer
Canadian Diabetes Association
Medical Research Council of Canada
National Surgical Advisor - Digestive Disease Center of Excellence - The Humana Corporation 1986-1994
Alpha Omega Alpha - Washington University Chapter - Elected Faculty Member January 1988
Outstanding Profession/Scientific Employee - Federal Employee of the Year Award Program - St. Louis Federal Executive Board 1990
The Huddinge Hospital Transplant Lectureship - Annual Meeting of the Swedish Society for Medical Science, Stockholm, Sweden, December 1990
Council Member - Cell Transplantation Society 1992-Present
Council Member - International Pancreas & Islet Transplantation Association 1993-Present
Editorial Board
Cell Transplantation 1992-1993
Transplantation Science

Committee Appointments:

Washington University Animal Studies Committee
Chairman 1991-1994
Washington University Medical Center Alumni Association
Committee Member 1991-1994
International Juvenile Diabetes Research Foundation Medical Science Review Committee 1990-1993
UNOS Pancreas Subcommittee Member 1991-1995
American Society of Transplant Surgeons Program and Publications Committee 1989-1991
Academic Freedom and Tenure Hearing Committee
Member 1985-1991
Washington University Committee on the Humane Care of Laboratory Animal Member
Member 1984-1990

Operating Room Technician Program
Forest Park Community College
Advisory Committee 1976-1995
Chairman 1986-1995
Mid-America Transplant Association
Member Professional Advisory Board 1985-1995
American Cancer Society Institutional Research Grants
Washington University Committee for Cancer Research
Member 1979-1989
Chairman 1982-1989
"Health Views" - Editorial Advisory Board
Member 1984-1988
American Diabetes Association, St. Louis Chapter
Research Committee 1985-1988
Department of Surgery Animal Facility
Director 1980-1984
Washington University Faculty Senate
Member 1981-1983
Executive Committee of the Faculty Council
Member 1982-1984
Clinical Sciences Research Building Animal Surgery Suite
Director 1984-1985
Department of Engineering Master Degree Thesis Review Committee:
1979-John Bergen - "Kinetics of Insulin Secretion from Pancreatic Islets of Langerhans and Development of Islet Transplantation Chambers"
1980-Paul Aegerter - "Microencapsulation of Living Cells to Prevent Immunological Response"
1983-Shiow Meei Lin - "Testing of a Mathematical Model for Islet Transplantation Chambers"
1987-Donna Wilkinson - "Coating of Live Cells with Polysaccharide Derivatives"
1989-Mary Blanchard - "Quantification of Low Concentrations of Polysaccharide Derivatives and Their Effect on Cell Viability"
1990-Ph.D. Thesis Review, Donna Hawk-Reinhard - "Purification of Pancreatic Islets of Langerhans Using Cell Electrophoresis"
St. Louis VAMC Committees
Comprehensive Planning Committee
Chairman 1988-1990
Administrative Executive Board 1988-1990
Professional Standards Board 1988-1990
Research Committee 1988-1994
District Planning Board 1988-1990

Barnes Hospital Committees
- Chaplaincy Committee 1992-1994
- Emergency Room Committee 1978-1984
- Search Committee for ER Director 1978-1984
- Patient Education Parent Committee 1979
- Surgery Patient Education Subcommittee Chairman 1981-1988

Tissue Culture Association
- Publicity Chairman 1980

Invited Presentations, Selected:

The Kroc Foundation
- Islet Transplantation Workshop 1974
- Islet Transplantation Workshop 1979
- Islet Transplantation Workshop 1982

National Institutes of Health
- National Conference on Diabetes 1979
- National Conference on Diabetes 1983

Juvenile Diabetes Foundation
- Conference on Research Tissue 1981
- National Meeting, Keynote Speaker 1984
- International Scientific Research Conference 1985

German Diabetes Association, Giessen, West Germany
- Islet Transplantation Workshop 1980
- Islet Transplantation Workshop 1989

American Society of Artificial Organs
- Annual Meeting - Keynote Speaker 1983
- Session Co-Chairman 1987

International Symposium on Organ Transplantation in Diabetes
- The Hague, Netherlands 1983

International Symposium on Kidney and Pancreas Transplantation
- Perugia, Italy 1984

International Islet Transplantation Workshop
- Canberra, Australia 1984

XII Congress of the International Diabetes Federation
- Madrid, Spain 1985

XIII Congress of the International Diabetes Federation
- Sydney, Australia 1989

National Disease Research Interchange
- Human Tissue Conference 1985
- Human Tissue Conference 1986
- Human Tissue Conference 1987
- Human Tissue Conference 1990

National Disease Research Interchange - Chairman of Task Force on "Biohazard and Contamination in the Use of Human Tissue and Organs"
Philadelphia, PA 1988
American Diabetes Association National Meeting - Session Co-Chairman for "Forms of Therapy" 1986
Visiting Scientist Program - University of Kansas Diabetes Center
Kansas City, Kansas 1986
Immunology of Diabetes Symposium - Member of International Advisory Committee
Edmonton, Canada 1986
International Symposium on Complications of Diabetes
The Hague, Netherlands 1986
Visiting Professorship - Department of Surgery - University of Minnesota
Minneapolis, Minnesota 1986
May 8th Endocrine Days
Victoria, British Columbia 1987
Second Annual Visiting Professorship in Diabetes - University of Wisconsin
Madison, Wisconsin 1987
First International Course on Transplantation
Venice, Italy 1987
Progress in Organ Transplants, Tissue Replacements and Implants
Sponsored by Biomedical Business International, New York 1987
Josiah Brown Memorial Symposium on Pancreas Beta Cell Transplantation
Los Angeles, California 1987
Seventh Workshop of the AIDSPIT Study Group
Igls, Austria 1988
First international Congress on Pancreatic and Islet Transplantation
Stockholm, Sweden 1987
Thirty-Fourth Annual Meeting of ASAIO, Invited Speaker "Modern Treatment of Insulin Dependent Diabetes"
Reno, Nevada 1988
Sixth Gordon Research Conference on Drug Carriers in Biology and Medicine
Plymouth, New Hampshire 1988
XII International Congress of the Transplantation Society
Sydney, Australia 1988
Second International Congress on Pancreatic and Islet Transplantation
Minneapolis, Minnesota 1989
Biology of Tissue Transplantation Symposium
Bethesda, Maryland 1989
Ninth Workshop of the AIDSPIT Study Group
Igls, Austria 1990
Society for Surgery of the Alimentary Tract Postgraduate Course, "Medical Aspects of Transplantation of the Liver, Pancreas and Intestine"
San Antonio, Texas 1990

Moderator for Pancreas Transplantation Scientific Session - American Society of Transplant Surgeons
Chicago 1990
UCLA Symposium on Molecular & Cellular Biology, "Tissue Engineering"
Keystone, Colorado 1990
The Huddinge Hospital Transplant Lectureship Annual Meeting of the Swedish Society for Medical Science
Stockholm, Sweden 1990
Third International Congress on Pancreatic and Islet Transplantation - Moderator and Plenary Speaker
Lyon, France 1991
European Association for the Study of Diabetes - Plenary Speaker
Dublin, Ireland 1991
Visiting Professor - University of Wisconsin
Madison, Wisconsin 1991
Moderator for Clinical Transplantation-Pancreas and Islets - XVIth International Congress of the Transplantation Society
Paris 1992
American Diabetes Association 53rd Annual Meeting - Plenary Speaker
Las Vegas, Nevada 1993
Fourth International Congress of Pancreas and Islet Transplantation - Plenary Speaker
Amsterdam 1993
IVth Joint Meeting of the Lawson Wilkins Pediatric Endocrine Society and the European Society for Pediatric Endocrinology - Symposium Speaker
San Francisco 1993
American Association for Clinical Chemistry
New York 1993

Publications

Abstracts:

1. Ballinger, W.F., Lacy, P.E., Scharp, D.W. Kemp, C.B., Knight, M. - Isografts and allografts of pancreatic islets in rats. Brit. J. Surg. 60:313, 1973

2. Kemp, C.B., Knight, M.J., Scharp, D.W., Lacy, P.E., Ballinger, W.F. - Islets of Langerhans injected into the portal vein of the diabetic rat. South African Journal of Surgery 11:135, 1973

3. Kemp, C.B., Knight, M.J., Scharp, D.W., Lacy, P.E., Ballinger, W.F. - Proceedings: Implantation of pancreatic islets into the portal vein of diabetic rats. Brit. J. Surg. 60:907, 1973

4. Scharp, D.W., Kemp, C.B., Knight, M.J., Murphy, J., Newton, W., Ballinger, W.F., Lacy, P.E. - Long term results of portal vein islet isografts and allografts in the treatment of Streptozotocin induced diabetes. Diabetes 23:359, 1974

5. Scharp, D.W., White, D.J., Ballinger, W.F., Lacy, P.E. - Transplantation of intact islets of Langerhans after tissue culture. In Vitro 9:364, 1974

6. Knight, M.J., Scharp, D.W., Kemp, C.B., Nunnelley, S.B., Ballinger, W.F., Lacy, P.E. - Cryopreservation of pancreatic islets. European Surgical Research 6(1):89, 1974

7. Ballinger, W.F., Murphy, J.J., Scharp, D.W., Hirshberg, G.E., Karl, R.C., Lacy, P.E. - Isolation and preservation of human islets of Langerhans for transplantation in the treatment of diabetes. European Society for Exp. Surg., Tenth Congress 1975

8. Griffith, R.C., Scharp, D.W., Ballinger, W.F., Lacy, P.E. - A morphologic study of intrahepatic portal vein islet isografts. Diabetes 34(2):419, 1975

9. Dodi, G., Scharp, D., Feldman, S., Maresca, B., Ballinger, W., Lacy, P. - Treatment of exocrine pancreatic dysfunction in diabetic rats by islet transplantation. European Surgical Research 9(1):98, 1977

10. Scharp, D.W., Merrell, R.C., Feldman, S., Ruwe, E., Feldmeier, M., Ballinger, W., Lacy, P. - Long term culture of islets of Langerhans utilizing a rotational culture method. In Vitro 13:174, 1977

11. Scharp, D., Krupin, T., Waltman, S., Oestrich, C., Feldman, S., Ballinger, W., Becker, B - Relationship of abnormal insulin release to fluorophotometry in experimental diabetes. Diabetes 27(2):435, 1978

12. Scharp, D.W., Merrell, R.C., Feldmeier, M.M., Downing, R., Ballinger, W.F. - Pseudo-islet formation and culture from canine isolated pancreatic cells. In Vitro 15:216, 1979

13. Rajotte, R.V., Scharp, D.W., Downing, R. Molnar, G.D., Ballinger, W.F. - The transplantation of frozen-thawed rate islets transported between centers. Diabetes 28:377, 1979

14. Downing, R. Scharp, D.W., Grieder, M., Ballinger, W.F. - Mass isolation of islets of Langerhans from the dog pancreas. Diabetes 28:426, 1979

15. Feldman, S.D., Scharp, D.W., Lacy, P.E., Ballinger, W.F. - Fetal pancreas isografts, cultured and uncultured to reverse Streptozotocin induced diabetes mellitus. The Association for Academic Surgery 12:116, 1979

16. Grieder, M.H., DeSchryver-Kecskemeti, K. Gingerich, R.L., Scharp, D.W. - In vitro studies using canine pseudo-islets and rat antrum cultures as models. UCLA Symposium, December 3, 1979

17. Scharp, D.W., Feldmeier, M.M., Rajotte, R.V., DeSchryver, K., Bell, M. - Human pseudo-islet formation, culture and preservation. Diabetes 29(2):18A, 1980

18. Gingerich, R.L., Scharp, D.W., Grieder, M.H., Dye, E.S., Mousel, K.A. - A new in vitro model to study secretion and biochemistry of pancreatic polypeptide (PP). Diabetes 29(2):30A, 1980

19. Bergen, J.F., Mason, N.S., Scharp, D.W., Sparks, R.E. - Insulin inhibition of islets in transplantation chambers. Presented at International Society for Artificial Organs Meetings, Paris, July 8-10, 1981

20. Sparks, R.E., Mason, N.S., Finley, T.C., Scharp, D.W. - Development, testing and modeling of an islet transplantation chamber, ASAIO Meetings, Chicago, April, 1982

21. Long, J.A., Adair, W.S., Scharp, D.W. - Hybridoma production against pancreatic cells. Diabetes 31(2):20A, 1982

22. Scharp, D.W., Hirshberg, G., Long, J.A. - The effect of islet dosage and time on rat portal vein isografts. Diabetes 31(2):162A, 1982

23. Scharp, D.W., Lacy, P.E. - The isolation and alteration of islet tissue for transplantation. The Tissue Culture Association Meeting, San Diego, June, 1982. In Vitro Suppl. 1, 1982

24. Long, J.A., Adair, W.S., Scharp, D.W. - An immunological approach to islet cell purification. J. Cell Biol. 95:4061, 1982

25. Sparks, R.E. Mason, N.S., Finley, T.C., Scharp, D.W. - Design of islet transplantation chambers giving a normal glucose tolerance test. ISAO Meetings, Kyoto, Japan, November, 1983

26. Sparks, R.E., Mason, N.E., Finley, T.C., Scharp, D.W. - Islet transplantation chamber models - assumption for insulin generation and glucose diffusion. International Symposium on Organ Transplantation in Diabetes, The Netherlands, September, 1983

27. Sparks, R.E., Mason, N.S., Scharp, D.W. - Some present directions in research on tissue transplantation chambers. International Conference on Artificial Organs, Glasgow, Scotland, September, 1983

28. Sparks, R.E., Mason, N.S., Finley, T.C., Scharp, D.W. - "A distributed source-model for hybrid artificial pancreas", presented by ASAIO, Toronto, Ontario, Canada, April, 1983

29. Scharp, D.W., Feldmeier, M.M., Olack, B.J., Swanson, C.J., O'Shaughnessey, S.F. - Electrophoretic purification of islet cells for transplantation. Diabetes 33(1):179A, 1984

30. Scharp, D.W., Rajotte, R.V., Kneteman, N.M., Lacy P.E. - Zero gravity electrophoresis of islet cells. 10th International Congress of the Transplantation Society Meeting, Minneapolis, August, 1984

31. Scharp, D.W., Lacy, P.E. - Human islet isolation and transplantation. Diabetes 34(1):5A, 1985

32. Kneteman, N.M., Alderson, D., Scharp, D.W. - Canine pancreatic islet allotransplantation: dose adjusted cyclosporine A vs azathioprine - steroid, Diabetes 34(1):62A, 1986

33. Alderson, D., Kneteman, N.M., Scharp, D.W. - The isolation of purified human islets of Langerhans. Diabetes 34(1):81A, 1986

34. Corlett, M.P., Fonseca, P., Scharp, D.W. - Detrimental effect of warm ischemia on islet isolation in rats and dogs with protection by oxygen free radical scavengers. Diabetes 36(1):223A, 1987

35. Scharp, D.W., Lacy, P.E., Finke, E.H., Olack, B.J. - Seven day culture of Ficoll purified human islets. Diabetes 36(1):222A, 1987

36. Corlett, M.P., Scharp, D.W. - Effect of warm ischemia on islet isolation in rats and dogs. The Association for Academic Surgery, Orlando, Florida, November 1-4, 1987

37. Misler, S., Gee, W., Scharp, D., Manchester, J., Falke, L. - Metabolically regulated potassium channels in human islet cells. Diabetes 37(1):6A, 1988

38. Atkinson, M.A., Maclaren, N.K., Riley, W.J., Scharp, D.W., and Holmes, L. - M_r 64,000 autoantibodies (64KA) predict insulin dependent diabetes (IDD). Diabetes 37(1):98A, 1988

39. Scharp, D.W., Lacy P.E., Ricordi, C., Boyle, P., Santiago, J.V., Gingerich, R.L., Anderson, C.B., Flye, M.W. - Human islet transplantation in patients with Type I diabetes. Presented at XII International Congress of the Transplantation Society, Sydney, Australia, August 14-19, 1988

40. Riley, W.J., Maclaren, N.K., Atkinson, M.A., Scharp, D.W., Lacy, P.E. - Islet autoantibodies in "prediabetes". Ninth International Workshop on the Immunology of Diabetes, Satellite Symposium on Beta Cell Destruction and Restoration in Type I Diabetes. November 27-29, Sydney, Australia, 1988

41. Atkinson, M.A., Maclaren, N.K., Riley, W.J., Scharp, D.W., Holmes, L. - The natural history and predictive value of M_r 64,000 islet cell antigen. Ninth International Workshop on the Immunology of Diabetes, Satellite Symposium of the International Diabetes Federation, Melbourne, Australia, 1988

42. Scharp, D.W., Chern, H.T., McCullough, C.S., Gingerich, R.L. - Lack of glucose induced insulin responsiveness in canine islets. 49th Annual Meeting of the American Diabetes Association, Detroit, Michigan, June 3-6, 1991. Diabetes 38(1):209A, 1989

43. Chern, H.T., Scharp, D.W., McCullough, C.S. - Cryogenic storage of purified canine islets. 49th Annual Meeting of the American Diabetes Association, Detroit, Michigan, June 3-6, 1989. Diabetes 38(1):246A, 1989

44. Callery, M.P., Ricordi, C., Scharp, D.W., Kamei, T., Lacy, P.E., Flye, M.W. - Hepatic insufficiency following portacaval shunting is prevented by prior intraportal pancreatic islet autotransplantation. The Society of University Surgeons, Baltimore, Maryland, 1989

45. Scharp, D.W., Corlett, M.P., Ricordi, C., Lacy, P.E. - Microbial contamination risk in human islet isolation. Study Group of the European Association for the study of Diabetes, Igls, Austria, 1989

46. Ricordi, C., Lacy, P.E., Socci, C., Finke, E.H. Dye, E., Olack, B.J., Swanson, C., Scharp, D.W. - Pancreatic islet isolation in mammals: Developments of the automated method. Study Group of the European Association for the Study of Diabetes, Igls, Austria, 1989

47. Callery, M.P., Kamei, T., Flye, M.W., Scharp, D.W. - Intrahepatic islet autografts in dogs function despite completely diverting portacaval shunt (PCS). Second International Congress on Pancreatic and Islet Transplantation, Minneapolis, Minnesota, 1989

48. McCullough, C., Chern, H.T., Scharp, D.W., Swanson, C.J., Olack, B.J., Davis, C.G. - Influence of transplant site on efficiency of canine islet cell auto-transplantation. Second International Congress on Pancreatic and Islet Transplantation, Minneapolis, Minnesota, 1989

49. Scharp, D.W. - islet Preservation - Short term for quality control testing. Presented at The Third International Conference on The Use of Human Cells, Tissues and Organs in Research. Washington, D.C., September 17-18, 1998

50. Falqui, L., Kowalski, L., Bittner, P., Olack, B.J., Scharp, D.W., Lacy, P.E. - Immunosuppressive regimens to prolong survival of islet xenografts transplanted across a wide species barrier (dog to mouse). Ninth Workshop of the AIDSPIT Study Group, Igls, Austria, January, 1990

51. Scharp, D.W., Lacy, P.E., Santiago, J.V., Boyle, P.J., Weide, L, Falqui, L., Marchetti, P, Gingerich, R.L., Cryer, P.E., Jaffe, A.S., McCullough, C.S., Anderson, C.B., Flye, M.W. - A clinical trial of islet transplantation. Ninth Workshop of the AIDSPIT Study Group, Igls, Austria, January, 1990

52. Marchetti, P., Falqui, L., Scharp, D.W., Lacy, P.E. - An improved method for large scale isolation of pure, viable islets of Langerhans from the adult porcine pancreas. Ninth Workshop of the AIDSPIT Study Group, Igls, Austria, January 1990

53. Scharp, D.W., Lacy, P.E., Santiago, J.V., McCullough, C.S., Weide, L.G., Falqui, L., Marchetti, P. Gingerich, R.L., Cryer, P.E., Jaffe, A.S., Anderson, C.B., Flye, M.W. - Preliminary results of clinical trials of human islet transplants. American Society of Transplant Surgeons 16th Annual Scientific Meeting, Chicago, Illinois, May 30-June 1, 1990

54. Marchetti, P., Scharp, D.W., Finke, E.H. - The isolation, function and xenotransplantation of porcine islets of Langerhans (PIL). 50th Annual Meeting of the American Diabetes Association, Atlanta, Georgia, June 16-19, 1990

55. Scharp, D.W., Lacy, P.E., McCullough, C.S., Weide, L., Boyle, P., Santiago, J.V., Gingerich, R., Cryer, P., Anderson, C.B., Flye, M.W. - Intraportal human islet transplants. XIII International Congress of The Transplantation Society, San Francisco, California, August 19-24, 1990

56. Finke, E.H., Marchetti, P., Swanson, C.J., Falqui, L., Lacy, P.E., Scharp, D.W. - Large scale isolation, function and transplantation of islets of Langerhans. XIII International Congress of the Transplantation Society, San Francisco, California, August 19-24, 1990

57. Olack, B.J., Swanson, C.J., McLear, M., Longwith, J., Scharp, D.W., Lacy, P.E. - Islet purification using Euroficoll gradients. XIII International Congress of the Transplantation Society, San Francisco, California, August 19-24, 1990

58. Scharp, D.W. - Clinical Trials. Presented at The Third International Conference on The Use of Human Cells, Tissues and Organs in Research. Washington, D.C., September 17-18, 1990

59. Swanson, C.J., Olack, B.J., Marchetti, P., Scharp, D.W. - Long-term metabolic and hormonal changes after intrasplenic canine islet transplantation. Fourteenth International Diabetes Federation Congress, Washington, D.C., June 23-28, 1991

60. Olack, B.J., Marchetti, P., Swanson, C.J., Kneteman, N.M., Scharp, D.W. - No adverse effects of Cyclosporine A on either oral or intravenous glucose tolerance tests in Transplanted Dogs. Fourteenth International Diabetes Federation Congress, Washington, D.C., June 23-28, 1991

61. McLear, M., Marchetti, P., Gingerich, R., Scharp, D.W. - The function of isolated human islets In Vitro. Fourteenth International Diabetes Federation Congress, Washington, D.C., June 23-28, 1991

62. Marchetti, P., Swanson, C.J., Olack, B.J., McCullough, C.S., Scharp, D.W. - The effect of islet transplant site and mass on long-term metabolic function of canine islet autografts. Fourteenth International Diabetes Federation Congress, Washington, D.C., June 23-28, 1991

63. Olack, B.J., Hill, A.C., Scharp, D.W., Lacy, P.E. - Automated purification of canine islets. Third International Congress on Pancreatic and Islet Transplantation, Lyon, France, June 6-8, 1991

64. McLear, M., Marchetti, P., Finke, E., Gerasimidi, A., Scharp, D.W., Lacy, P.E. - Porcine islet function: effects of arginine, leucine, and butyrate. Third International Congress on Pancreatic and Islet Transplantation, Lyon, France, June 6-8, 1991

65. Swanson, C.J., Marchetti, P., Newton, M., Scharp, D.W. - Effects of insulin therapy on glucose metabolism and insulin secretion in dogs and pancreatic islet autografts. Third International Congress on Pancreatic and Islet Transplantation, Lyon, France, June 6-8, 1991

66. Scharp, D.W., Lacy, P.E., Longwith, J.E. - Bacteriologic risks of human islet isolation for transplantation. Third International Congress on Pancreatic and Islet Transplantation, Lyon, France, June 6-8, 1991

67. Marchetti, P., Pfiffner, K., Scharp, D.W., Lacy, P.E., Gerasimidi, A., Finke, E.H. - Successful cryopreservation of porcine pancreatic islets (PI). Third International Congress on Pancreatic and Islet Transplantation, Lyon, France, June 6-8, 1991

68. Marchetti, P., Swanson, C.J., Finke, E.H., Gerasimidi, A., Scharp, D.W., Lacy, P.E. - In Vitro function and xenotransplantation of long-term (3 weeks) cultured porcine islets of Langerhans. First international Congress on Xenotransplantation. Minneapolis, Minnesota, August 25-28, 1991

69. Marchetti, P., Giannarelli, Scharp, D.W., di Carlo, A., McLear, M., Lacy, P.E., Navalesi, R. - Metformin potentiates glucose stimulated insulin release: a study on diabetic patients and isolated human islets. European Association for the Study of Diabetes 27th Annual Meeting, Dublin, Ireland, September 10-14, 1991

70. Scharp, D.W., Marchetti, P., Swanson, C.J., Olack, B.J., Newton, M. - The effect of implantation site and islet mass on long-term survival and metabolic function of pure canine islet autografts. European Association for the Study of Diabetes 27th Annual Meeting, Dublin, Ireland, September 10-14, 1991

71. Marchetti, P., Finke, E., Pfiffner, K., Swanson, C., Gerasimidi-Vazeou, A., Olack, B., Scharp, D.W., Lacy, P.E. - Cryogenic storage of purified porcine islets. Presented at Study Group of the European Association for the Study of Diabetes, Igls, Austria, January 26-28, 1992

72. Scharp, D.W., Olack, B.J., Pfiffner, K., Gerasimidi-Vazeou, A., Finke, E.H., Lacy, P.E. - Preserving human islets prevents their rejection as xenografts. Presented at the First International Congress of the Cell Transplant Society, Pittsburgh, Pennsylvania, May 31-June 3, 1992

73. Marchetti, P., Scharp, D.W., Olack, B.J., Swanson, C.J., Bier, D., Cobelli, C., Toffolo, G., Newton, M., Navalesi, R. - Glucose metabolism, insulin sensitivity and glucagon secretion in dogs with intraportal or intrasplenic islet autografts. Presented at the First International Congress of the Cell Transplant Society, Pittsburgh, Pennsylvania, May 31-June 3, 1992

74. Marchetti, P., Scharp, D.W., Olack, B.J., Swanson, C.J. - Prediction of islet autograft failure by fasting glucagon:insulin ratio. Presented at the XIV International Congress of the Transplantation Society, Paris, France, August 16-21, 1992

75. Flavin, K.S., Scharp, D.W., Lacy, P.E. - Pre-islet transplant evaluation of psychiatric status using the National Institute of Mental Health (NIMH) Diagnostic Interview Schedule (DIS). Presented at the American Diabetes Association Meetings, San Antonio, Texas, June 20-23, 1992

76. Hawk-Reinhard, D.R., Scharp, D.W., Sparks, R.E. - Buffer solution temperature and velocity profiles in vertical micro-electrophoresis. Presented at American Institute of Chemical Engineers, New York, NY, November, 1992

77. Marchetti, P., Scharp, D.W., Finke, E., Pfiffner, K., Swanson, C., Gerasimidi-Vazeou, A., Olack, B., Navalesi, R., Lacy, P.E. - Insulin release upon perifusion and xenotransplantation of cryopreserved porcine islets. Presented at European Association for the Study of Diabetes, Igls, Austria, January 1992

78. di Carlo, A., Scharp, D.W., Gingerich, R.L., Ansara, M., Olack, B., Navalesi, R., Swanson, C. - Effect of culture and cryopreservation on insulin release from isolated, perifused human islets. Hormone and Metabolic Research 25:52, 1993

79. Marchetti, P., Scharp, D.W., Finke, E., Swanson, C.J., Gerasimidi-Vazeou, A., Olack, B.J., Navalesi, R., Lacy, P.E. - Prolongation of pig-to-mouse islet xenograft survival. Hormone and Metabolic Research 25:62, 1993

80. Hegre, O., Laurance, M., Harvey, J., Dionne, K., Appel, M., Scharp, D., Lacy, P.E. - Species variation in insulin secretory capacity of isolated islets. Hormone and Metabolic Research 25:58, 1993

81. Marchetti, P., Scharp, D.W., McLear, M., Finke, E.H., Olack, B.J., Swanson, C.J., Giannarelli, R. Navalesi, R., Lacy, P.E. - Pulsatile insulin secretion from isolated human pancreatic islets. Presented at the 53rd Annual Meeting of the American Diabetes Association Meeting, Las Vegas, Nevada, June, 1993

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